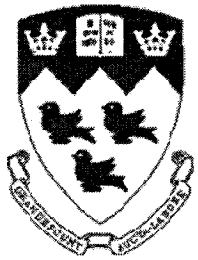

Epigenetic Programming by Maternal Behaviour

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Your file Votre référence
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To my Mum,

who contributed so much to what I am today,

and who also provided many fields for my learning.

ABSTRACT

Individual differences in gene expression and behaviour are the result of an interaction between a given set of genes and a variety of environmental conditions. The study of epigenetics focuses on the nature of this interaction. The early developmental period in the life of a mammal is exquisitely sensitive to environmental cues. One critical question is how is the genome capable of adapting to these developmental or environmental signals? Building on an established rodent model, in which the effects of maternal care have been demonstrated to regulate the development and expression of neurobiological and behavioural measures in offspring as adults, the current project concerns how events during the first week of life can have sustained effects on gene expression, which persist throughout the life of the organism.

The research presented in this thesis demonstrates how one facet of mothering style leads to a cascade of molecular and cellular changes, resulting in life-long alterations in the nature of stress responses and neuron survival. Frequent licking/grooming by rat mothers alters DNA methylation of the hippocampal glucocorticoid receptor (GR) gene and acetylation of histones early in life, providing a mechanism for these permanent changes in stress responses. Through postnatal cross-fostering studies, I was able to directly study how an identical gene within the same rat strain is expressed and regulated under the different developmental environments and how such effects on gene expression persist through life. I have also examined the potential for reversibility of the long-term consequences of postnatal environment and have demonstrated that both GR levels and the nature of stress responses exhibit a high degree of plasticity in adulthood in response to both pharmacological intervention and dietary amino-acid supplementation. These results demonstrate that the epigenomic marks established early in life through a behavioural mode of programming, are dynamically maintained and potentially reversible in the adult brain. These results contrast with the very dogmatic view that the genome is rendered fixed and immutable. I next questioned the global effects of early-in-life experience on the hippocampal transcriptome and anxiety-mediated behaviours in adulthood. Microarray analysis revealed > 900 different maternal care-responsive mRNA transcripts. These results suggest that effects of early

life experience have a stable and broad effect on the hippocampal transcriptome, which may play a role in the development of anxiety-mediated behaviours through life. Finally, both *in vivo* and *in vitro* studies show that maternal behaviour increases GR expression in the offspring via increased hippocampal serotonergic tone accompanied by increased histone acetylase transferase activity, histone acetylation and DNA demethylation mediated by the transcription factor NGFI-A.

In summary, this research demonstrates that an epigenetic state of a gene can be established through early-in-life experience, and is potentially reversible in adulthood. We predict that epigenetic modifications of targeted regulatory sequences in response to variations in environmental conditions might serve as a major source of variation in biological and behavioural phenotypes. In the case of GR, the resulting individual differences in behavioural and physiological responses to stress are thought to be a major risk factor for the development of psychiatric and physical illness. Thus, in addition to contributing to our understanding of how gene-environment interactions shape development, our work provides a mechanism that can be targeted for therapeutic intervention to potentially reduce the prevalence of these disorders.

RÉSUMÉ

Les différences individuelles dans l'expression de gènes et le comportement sont le résultat d'une interaction entre un ensemble de gènes et une variété de conditions environnementales. L'étude épigénetique se concentre sur la nature de cette interaction. La période précoce du développement dans la vie d'un mammifère est extraordinairement sensible aux conditions environnementales. Une question critique serait de savoir comment le génome peut s'adapter à ces signaux développementaux ou environnementaux? Sur la base d'un modèle établi de rongeur, dans lequel les soins maternels modulent le développement et l'expression de paramètres neurobiologiques et comportementaux de progénitures rendus à l'age adulte, le présent projet cherche à déterminer comment les événements pendant la première semaine de la vie peuvent avoir des effets soutenus sur l'expression de gènes, qui persisteront durant toute la vie de l'organisme.

La recherche présentée dans cette thèse démontre comment une facette du soin maternel mène à une cascade de changements moléculaires et cellulaires, ayant pour conséquence des changements permanents dans la nature des réponses de stress et dans la survie de neurones. Le lèchement/soins de toilettage fréquent de la portée par leur rate génitrice change la méthylation de l'ADN du gène codant pour le récepteur des glucocorticoïdes (GR) au niveau de l'hippocampe ainsi que l'acétylation des histones tôt dans la vie, ce qui fournis un mécanisme pour ces changements permanents en réponse à des stress. Par des études de croisement de portées entre les mères, je pouvais étudier directement comment un gène identique dans la même souche de rats est exprimé et régulé sous différentes conditions développementales et comment de tels effets sur l'expression de gènes persistent durant toute la vie. J'ai également examiné le potentiel pour la réversibilité des conséquences à long terme de l'environnement postnatal et démontré que les niveaux de GR et la nature des réponses à des stress montrent un degré élevé de plasticité chez l'adulte en réponse à l'intervention pharmacologique et au supplément diététique d'acides aminés. Ces résultats démontrent que les traits épigénomiques établis tôt dans la vie par une programmation par le comportement, sont dynamiquement maintenus et potentiellement réversibles dans le cerveau de l'adulte. Ces résultats diffèrent de la vue très dogmatique que le génome est

rendu fixe et immuable. Nous sommes par la suite questionnés sur les effets globaux de l'expérience précoce après la naissance sur le transcriptome de l'hippocampe et des comportements médiés par l'anxiété. L'analyse de biopuces d'ADN (*Microarray*) a indiqué que plus de 900 gènes sont sensibles aux soins maternels. Ces résultats suggèrent que les effets des premières expériences de la vie ont un effet stable et étendu sur le transcriptome de l'hippocampe, ce qui peut influencer le développement des comportements faces à l'anxiété durant la vie. En conclusion, les études *in vivo* et *in vitro* prouvent que le comportement maternel augmente l'expression des GR dans la progéniture via un tonus sérotonergique accru de l'hippocampe, accompagnée par des augmentations dans l'activité des acétylases d'histones, dans l'acétylation d'histones et dans la déméthylation de l'ADN relayé par le facteur de transcription NGFI-A.

En résumé, cette recherche démontre que l'état épigénétique d'un gène peut être établi par l'expérience des premiers moments de la vie, et est potentiellement réversible dans l'âge adulte. Nous prévoyons que les modifications épigénétiques de séquences de régulation cibles en réponse aux variations des conditions environnementales pourraient servir de source importante de variation dans les phénotypes biologiques et comportementaux. Dans le cas des GR, les différences individuelles dans les réponses comportementales et physiologiques face aux stress pourraient être un facteur de risque important dans le développement de maladies psychiatriques et physiques. Ainsi, en plus de contribuer à une meilleure compréhension sur la façon dont les interactions entre les gènes et l'environnement modulent le développement, notre travail fournit un mécanisme et une cible future pour une intervention thérapeutique visant à réduire potentiellement la prévalence de ces désordres.

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LIST OF ABBREVIATIONS

Units

AU – arbitrary unit

Å – Angstrom

bp – base pair

cm – centimetre

cpm – counts per min

°C – degrees centigrade

h – hour

K_d – dissociation constant

kDa – kilo Dalton

l – litre

µg – microgram

µl – microlitre

µM – micromolar

µm – micrometer/micron

mM – millimolar

min – minute

M – molar

ng – nanogram

% – percentage

pmol – picomolar

RPM – revolutions per min

sec – second

v – volts

w/v – weight per volume

Chemicals, molecules & sundry

1_7 – the seventh promoter site on exon one of the rat GR gene

3β HSD – three beta-hydroxy steroid dehydrogenase

3V – third ventricle

5-HT – five hydroxytryptamine (serotonin)

α – alpha

αα – amino acid

α_s – stimulatory alpha protein

A – adenine

A-II – angiotensin-two

AC – adenylate cyclase

ACTH – adrenocorticotropic Hormone

ADP – adenosine diphosphate

AIF – apoptosis inducing factor

AF-2 – activation function-2

AHP – after hyper-polarisation

AMPA – α-amino-3-hydroxy-5-methyl-

4-isoxazole propionic acid

ANOVA – analysis of variance

ANT – adenosine nucleotide transporter

AP 1/2 – activator protein one or two

APAF-1 – apoptosis activating factor-1

Arg – L-arginine

ASP – aspartic acid

ATP – adenosine triphosphate

AVP – arginine vasopressin

β – beta

BAD – BCL-2 associated death protein

BAX – BCL-2 associated protein-X

BCL-2 – B cell lymphoma-2

BDNF – brain derived neurotrophic factor

BnST – bed nucleus of the stria terminalis

C – cysteine

C-/COOH- – carboxy terminal

Ca^{2+} – calcium

CA – cornu ammonis

cAMP – cyclic adenosine 3′, 5′ monophosphate

CASPase – cysteinyl aspartate specific proteinase

CBG (Transcortin) – corticosteroid binding globulin

CBP – CREB binding protein

cDNA – complementary DNA

CeA – central amygdala

CNS – central nervous system

CpG – cytosine phosphate guanosine

CRE – CREB response element

CREB – cAMP response element binding protein

CREM – CRE modulator

CRH – corticotrophin releasing hormone

CRH-R_1 – CRH receptor one

Cyto-C – cytochrome carboxylase

DA – dopamine

DAG – diacylglycerol

DAT – dopamine transporter

dATP – deoxy adenosine triphosphate

DBD – DNA binding domain

ddH_2O – double deionised water

DHEA – dehydroepiandrosterone

DHEA-s – DHEA sulphate

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

dNTP – deoxynucleoside triphosphate

ε – epsilon

ED – embryonic day

ER-α – oestrogen receptor-alpha

ERE – oestrogen response element

EtBr – ethidium bromide

ETC – electron transfer chain

FA – fatty acid

FADD – FAS associated death domain

FAS – fatty acid synthase

γ – gamma

G – guanosine

GABA – γ amino butyric acid

GC – glucocorticoid

GDP – guanine diphosphate

GF – growth factor

GluR – glutamate receptor

GPCR – guanine protein coupled receptor

GR – glucocorticoid receptor

GRIP – GR-interacting protein

GRL – GR gene locus

GTP – guanine triphosphate

H – histone

HAT – histone acetylase transferase

LBD – ligand binding domain

HDAC – histone deacetylase

hGR – human GR

HMGCoA – 3-hydroxy-3-methyl glutarylcoenzyme-A

HPAA – hypothalamic pituitary adrenal axis

HRE – hormone response element

hsp – heat shock protein

HZ – heterozygous

ICER – inducible cAMP early repressor

IEG – immediate early gene

IL – interleukin

IP3 – inositol 1,4,5 triphosphate

κ – kappa

K^+ – potassium

KO – knockout

LDL – low-density lipoprotein

LG – licking and grooming

LLP – long-lasting potentiation

LPS – lipopolysaccharide

LTP – long-term potentiation

MBD – methyl CpG binding domain

MC – mineralocorticoids

ME – median eminence

MET – L-methionine

MeTase – methyltransferase

mGR – mouse GR

MPOA – medial preoptic area

MR – mineralocorticoid receptor

mRNA – messenger ribonucleic acid

MS – maternal separation

MTP – mitochondrial transition pore

N-/NH3- – amino terminal

Na^{2+} – sodium

NaCl – sodium chloride

NaCl (0.9 %) – saline

NF-κB – nuclear factor-kappa B

NGF – nerve growth factor

NGFI-A – NGF-inducible protein A

NMDA – N-methyl-D-aspartate

NR-1 – NMDA receptor-1

NTR – nuclear transcription regulator

$NTRK_1$ (TRKA) – neurotrophic tyrosine kinase receptor type-1

O_2 – Oxygen

OT – oxytocin

OTR – oxytocin receptor

P – probability (statistics)

PARP – poly ADP ribose polymerase

PCD – programmed cell death

PCR – polymerase chain reaction

pCREB – phosphorylated CREB

PIP_2 – phosphatidyl inositol 4, 5 bisphosphate

PKA – protein kinase-A

PKC – protein kinase-C

PLC – phospholipase-C

PND – post-natal-day

POMC – proopiomelanocortin

pPVN – parvocellular PVN

PS – phosphatidylserine

PVN – paraventricular nucleus

rGR – rat GR

RIP – receptor-interacting protein

RNA – ribonucleic acid

SAM – S-adenosyl-methionine

SAMS – sympatho-adreno-medullary system

SER – smooth endoplasmic reticulum

SHR – spontaneously hypersensitive rat

SNS – sympathetic nervous system

SON – supraoptic nucleus

SRC – steroid receptor co-activator

SRE – serum response element

SREBP – sterol regulatory element bind-

ing protein

T – thymine

T_3 – triidothyronine

Tdt – terminal deoxynucleotidyl transferase

TIF – transcription intermediary factor

Ta – annealing temperature

TMD – transmembrane domain

TNF – tumour necrosis factor

TRE – thyroid receptor response element

USV – ultrasonic vocalizations

UV – ultraviolet

VDAC – voltage-dependent anion channel

VTA – ventral tegmental area

WKY – Wistar Kyoto rat

Zn^{2+} – Zinc

Chapter 1
Introduction

A common theme across all forms of life is the ability to vary phenotype in response to environmental conditions, termed phenotypic plasticity (Agrawal, 2001). Indeed, the potential for phenotypic plasticity is apparent in mammals, reptiles, insects and even plant species (Mousseau and Fox, 1998; Qvarnstrom and Price, 2001; Rossiter, 1999). The primary objective of phenotypic plasticity is to fine-tune the development of specific biological systems to enhance the match between phenotype and environmental demand. Early postnatal life represents a period when external stimuli, whether they are permissive or aversive, can influence emotional and cognitive development in human and non-human primate offspring (Ainsworth and Brown, 1991; Ammerman, 1991; Suomi, 1997). There is significant experimental evidence to indicate the importance of maternal influences on subsequent performance both in terms of physiological functions and behaviour of the offspring (Denenberg, 1999; Ottinger and Tanabe, 1969; Ressler and Anderson, 1973). Importantly, the nature of mother-offspring interaction influences gene expression and the development of behavioural responses in the offspring, which remain stable from early development to the later stages of life.

Though the importance of maternal care in promoting the health, survival and cognitive development of offspring has been demonstrated across the mammalian kingdom (Gale and Martyn, 1996; Kraemer, 1997; Liu et al., 1997), the most extensively researched animal model of mother-infant interactions is the rat. The rat provides a practical model for examining the neural circuitry of complex behaviour *in vivo*. The rat lifespan (~26-32 months) allows for the study of multiple generations of multiple cohorts of offspring within a relatively brief length of time. Though less developed in the prefrontal cortex, the rat

brain shows a high degree of homology with the human brain. Furthermore, the same hormones and neurotransmitters that mediate neuroendocrine and behavioural responses in the rat have been shown to have similar, if not the same, functions in the human. Moreover, though the *form* of behaviour provided by the rat and human may differ considerably, the *function* of these behaviours remains similar.

Accordingly, such phenotypic variation reflects complex gene-environment interactions involving protein-DNA interactions with regions (promoters, enhancers, and suppressors) that regulate the expression of the coding regions of the genome. Indeed, the main challenge of a complex organism, such as the rat, is how to regulate the expression of a specific set of genes from a repertoire of genomic sequences. Gene expression, in part, is determined by the nuclear packaging of DNA, the chromatin structure. In addition to chromatin status, the genomic DNA is covalently modified by methylation. Chromatin structure and DNA methylation patterns are unique to each type of cell. Active chromatin is associated with hypomethylated DNA, whereas inactive chromatin is associated with hypermethylated DNA. Herein, we propose that chromatin conformation and DNA methylation form the dynamic interface between the ever changing environment and the fixed world of genetics, termed the epigenome.

At the most rudimentary level, this doctorial thesis concerns the elucidation of how the effects of early life experiences are rendered permanent through life. Fundamentally, this research demonstrates the relationship between genes and environment and illustrates how environment, acting through the epigenome, can alter long-term patterns in gene expression and behaviour. This project examines the neural mechanisms mediating epigenetic programming of stress responses by maternal behaviour. The following chapter provides an overview of the current literature relevant to understanding maternal behaviour and responses to stress in the rat.

Chapter 2
Review of the Literature

2.1. *Maternal Behaviour in the Rat*

In mammals, the primary care giver (most often the mother) serves as a direct link between the environment and the developing offspring. Maternal care involves a pattern of behaviours that provide a nurturing environment crucial for the survival of the neonate. From a developmental perspective, the early postnatal period in the rat is comparable to the third trimester in human pregnancy. In relation to the human, the rat brain is relatively immature at birth and continues to develop during the postnatal period. Thus, variations in mother-pup interaction serve to carry great importance for development of the young.

In the rat, parturition (birth) occurs most often during the light (inactive) period of the light:dark cycle on embryonic day (ED) 21-23 of pregnancy. Litter sizes vary among different strains of rats. Rat pups, like most rodents, are born blind, naked (except for small vibrissae on the snout) and with their ear canals closed and the external ear (pinna) flattened against the head. Furthermore, they are unable to urinate and defecate properly or maintain their own body temperature (Krinke, 2000). The mother-infant relation regulates physiological responses such as heart rate, sleep/wake cycle, thermoregulation, gastrointestinal activity and growth hormone production in the infant (Hofer, 1994).

Following birth, the dam initiates maternal behaviour towards the pups. Females are attracted to pups by their odours and emission of ultrasonic vocalizations (USVs) charterised by frequencies ranging between 30 and 90 kHz (Branchi et al., 2001; Hofer, 1996). The ontogenic development of rat pup USVs displays a characteristic time course starting around postnatal day (PND) 2-3, reaching a plateau level around PND 4-6 and then gradu-

ally decreases (Johansson-Wallsten et al., 1993). Moreover, rat pup USVs might reflect a general emotional distress caused by the absence of the mother and have been shown to elicit mother-pup interactions (Branchi et al., 2001; Hofer, 1996). Mother-pup contact occurs primarily within the context of a nest bout, which begins when the mother approaches the litter and gathers the pups under her; she then nurses her offspring, intermittently licking and grooming the pups, particularly the head and anogenital region (Figure 1) (Alberts and Leimbach, 1980; Fleming and Rosenblatt, 1974a). Nest bouts (~20 min) also result in a rise in maternal temperature, which has been suggested to determine the duration of the nest bout (Leon et al., 1978). Another important component of maternal behaviour is to protect the litter from conspecific intruders (Elwood, 1983; Rosenblatt, 1989). During the beginning of the lactating period, the mother spends approximately 80-85 % of the time with her litter. This time slowly declines as the pups mature to around 30 % by PND 21 (Grota, 1969; Leon et al., 1978).

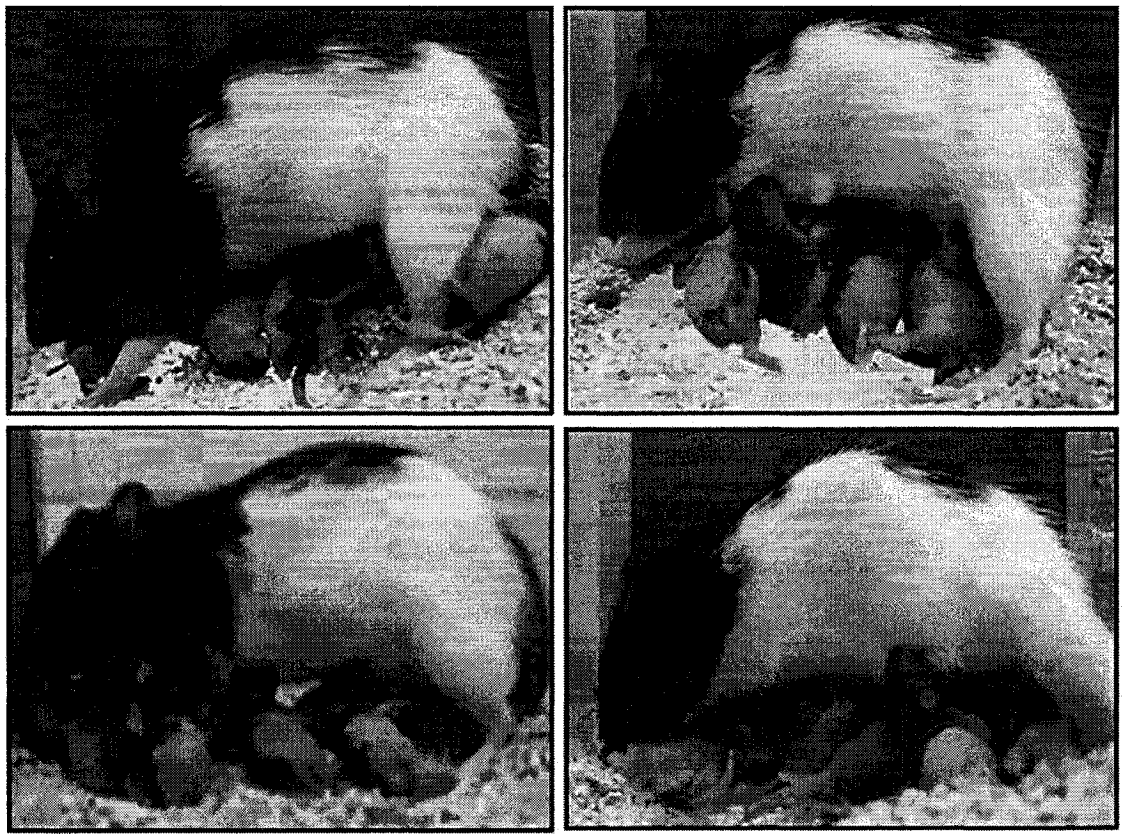

Figure 1: Four panels showing different nurturing arched-back postures of the female rat within a nest bout; with offspring gathered beneath her, facilitating the suckling of milk, the dam intermittently licks and grooms the pups (see text for details).

By retrieving and grouping the pups under her, the pups gain access to the dams ventrum for warmth (Croskerry et al., 1978; Leon et al., 1978) and the nipples to suckle milk (Stern and Johnson, 1990). The arched back nursing posture of the mother provides a source of tactile stimulation derived from 'nipple-switching' by the young offspring (Cramer et al., 1990; Thiels et al., 1990). Tactile stimulation also occurs through licking and grooming which behaviourally stimulates the pups, and in turn enhances their ability to attach to the nipples for food and thermoregulation (Sullivan et al., 1988a; Sullivan et al., 1988b). Though not all licking and grooming is anogenital, pup anogenital licking by dams serves a vital function: non-licked pups cannot defecate, and die. Interestingly, an ester, dodecyl-propionate isolated secreted from the preputial glands of the pup was found to regulate licking and grooming behaviour of the dam (Brouette-Lahlou et al., 1999).

Moreover, female rats display natural variations in the extent of maternal behaviour; i.e., either a high and low amount of licking and grooming and arched-back nursing (LG-ABN) towards their pups (Liu et al., 1997). Though an early study showed the dams interacted differently with male and female offspring (Moore and Morelli, 1979), a recent study indicated no such differences (Champagne et al., 2003). Dams stimulate reflexive urination and defecation by licking pup's anogenital areas until PND 14. In the second week of life licking and grooming contributes to the masculinity of the male pups (Moore, 1984). Licking and grooming enables the dam to reclaim essential ions and fluid lost during lactation. The mother actually ingests the pup urine, as much as 40-50 mls per day, which serves as a major source of sodium (Na^{2+}) (Gubernick and Alberts, 1983). Accordingly, mothers maintained on saline (0.9 % NaCl) in their drinking water show reduced anogenital licking (Gubernick and Alberts, 1983; Moore and Power, 1992). However, while this manipulation reduces anogenital licking, it does not affect body-licking (Moore and Power, 1992). Body licking is regulated by perioral somatosensory cues (Stern and Johnson, 1990). Thus, injections of a local anaesthetic (lidocaine) into the mystacial pads of the mother significantly reduces body-licking of the offspring (Stern and Kolunie, 1989).

In the beginning of the second week of life, the rat pups develop fur and, consequently, begin self-thermoregulation. By two weeks of age the pups open their eyes and ears, locomotor functions become more efficient and the pups begin to show increased

physiological autonomy and begin to explore their environment more frequently. The dam makes suckling progressively more difficult for the pups and so the young loose interest and by PND 21-25 begin to live more independently, a process termed weaning (Rosenblatt, 1989).

Puberty in male rats is hormonally dependent and occurs around PND 30-40. In the female rat, puberty is associated with the vaginal opening and the first pro-oestrus and the timing is apparently dependent more on body weight than on age. Vaginal opening in the female offspring occurs approximately on PND 33-42 with the body weight just above 100 g. About one week after the vaginal opening, when the body weight reaches approximately 120 g, the female starts to show regular oestrus cycles (Krinke, 2000). The fully sexually developed female is then able to mate with a sexually developed male and provide a nurturing environment for her own offspring.

In summary, maternal behaviour in the rat during the first weeks of life provides the nurturing environment that is crucial for survival of the young and allows the dam to meet the physiological demands of prolonged care of the offspring. Though lactating female rats display the full range of maternal behaviours previously described, this is not the case for virgin or pregnant rats. Adult virgin females exposed to pups will typically display avoidance behaviours, attempting to either hide the pups under bedding material or relocate to a position as far away from the pups as possible (Fleming, 1986; Fleming and Rosenblatt, 1974a). Females will even cannibalize pups upon initial exposure to them. However, exposure of females to pups repeatedly over the course of several consecutive days increases the propensity to engage in maternal behaviour (Rosenblatt, 1967). The latency in days of exposure to the induction of maternal behaviour ranges from three to sixteen days in virgin female rats (Fleming, 1986). Indeed, maternal-responsive behaviour in the rat has provided much insight into the anatomical and neurochemical mechanisms involved in regulating maternal care, which are described in the following section.

2.1.1. Regulation of Maternal Behaviour: The Maternal Brain Circuit

The central neuroanatomical circuitry in the female rat brain that mediates maternal-responsive behaviour is termed the maternal circuit (Figure 2) (Fleming, 1986; Numan and

Numan, 1994). Olfactory cues provide the rat with information regarding the characteristics of the environment for development and survival (i.e., food, mate and predation) (Cheal and Sprott, 1971). Through social olfaction, virgin female rats form an immediate aversion to young pups resulting in the observed latency in maternal-responsive behaviour, which varies across the oestrus cycle; di-oestrus females are significantly less maternal than pro-oestrus females (Gonzalez and Deis, 1986). However, the latency for maternal behaviour can be shortened by rendering the females without the sense of smell (anosmic) (Fleming and Rosenblatt, 1974b; Fleming and Rosenblatt, 1974c; Mayer and Rosenblatt, 1975). Olfactory cues from the pup stimulate neural projections connecting the main and accessory olfactory bulb to the amygdala, which is composed of the basolateral, medial and central nuclei (Ono et al., 1985). The medial amygdala sends neural projections to the hypothalamus, resulting in increased levels of oestrogen in the medial preoptic area (MPOA). The steroid-bound form of oestrogen receptor (ER) interacts with sequence-specific genomic response elements (ERE) and regulates transcriptional activity (Carson-Jurica et al., 1990; Loven et al., 2001; Nilsson et al., 2001). Therefore, the action of oestrogen on the ER alters the expression of cellular mRNA transcripts and proteins and influence fundamental properties of MPOA neurones, including oestrogen sensitivity. The induction of maternal behaviour has been primarily associated with the endocrine functions of oxytocin (OT) (Fahrbach et al., 1985). Intracerebrovascular (ICV) infusions of OT reduce latencies to maternal behaviour in oestradiol-primed female rats (Fahrbach et al., 1984; Pedersen and Prange, 1979). Conversely, oxytocin receptor (OTR) antagonists infused centrally can inhibit maternal behaviour through blockade of OTRs in the hypothalamus and MPOA (Pedersen et al., 1985; van Leengoed et al., 1987). Importantly, OT infused females exhibit increased levels of maternal licking and grooming of the offspring (Fahrbach et al., 1984). MPOA expression of the transcription factor ER-α protein colocalises with OTR mRNA transcript expression in oxytocinergic neurones. Furthermore, knockout studies show that the ER-α is essential for induction of OTR by oestrogen (Young et al., 1998).

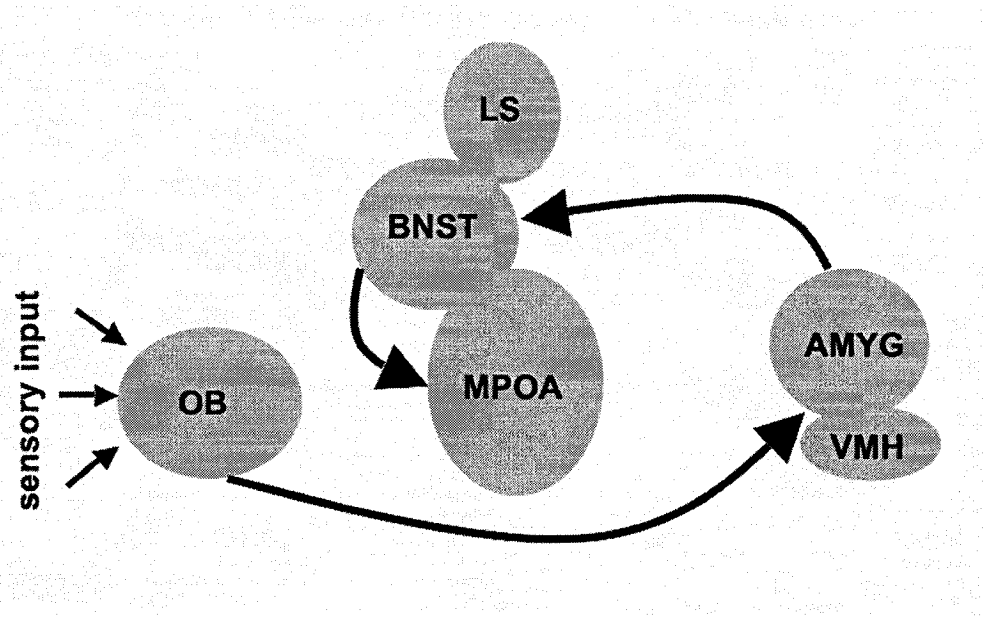

Figure 2: Functional anatomy of the maternal brain circuit (see text for details). Abbreviations denote the following: AMYG, amygdala; BNST, bed nucleus of the stria terminalis; LS, lateral septum; MPOA, medial preoptic area; OB, olfactory bulb; VMH, ventral medial nucleus of the hypothalamus [adapted from (Numan, 1988)].

The MPOA sends projections to the ventral tegmental area (VTA), thus connecting the maternal circuit to the mesolimbic dopamine (DA) system (Numan and Smith, 1984; Robbins and Everitt, 1996). OTRs in the MPOA may activate an oxytocinergic MPOA-VTA projection. Mesolimbic DA is a potential downstream target for oxytocinergic regulation. DA levels in the nucleus accumbens shell are associated with variations in mother-pup interactions (Hansen et al., 1993). More recently, it was shown that an increase in levels of DA precedes the onset of a licking and grooming bout (Champagne et al., 2004). The average number of seconds between the onset of the rise in DA and the onset of licking and grooming is 37.4 ± 7.0 sec, ranging from 5 to 230 sec. Moreover, the duration and magnitude of the DA signal in the nucleus accumbens shell increases directly in relation to the duration of the licking and grooming bout. The effects of DA on maternal behaviour are enhanced by decreased DA transporter (DAT) levels within the nucleus accumbens shell. The DA signal in the nucleus accumbens shell could serve to increase ER-α expression, and as a consequence OTR expression in the MPOA to further increase maternal behaviour.

The literature described above provides a general framework of the anatomy and neurotransmitters of the maternal brain. However, during the postnatal period, the infant is

dependent on the mother not only for nursing and protection but also for its own brain development. Thus, exploring environmental influences during the perinatal period can help provide a greater understanding of early life influences on the development of aberrant adult behaviour.

2.1.2. Neonatal Handling & Maternal Separation

Neonatal-handling is a simple animal model of stress that consists of a physical stimulus with a strong psychological component. The handling procedure involves removing the dam and then rat pups from their cage, placing the pups together in a small container, and returning the animals fifteen minutes later to their cage and their mother. The manipulation is performed daily during the first ten days of life. In unison, the artificial and non-specific nature of the handling paradigm is unsettling (Daly, 1973). However, neonatal-handling does change the mother-pup interaction (Villescas et al., 1977). Mothers of handled pups spend the same amount of time with their litters as mothers of non-handled pups; however, mothers of handled litters have shorter, but more frequent nest bouts.

Brief bouts of neonatal-handling (15-20 min) and extended periods of maternal separation (≥ 3 h) have disparate effects on neuroendocrine and behavioural responses to stress in the offspring that contribute to cognitive development later in life (Levine, 1967; Plotsky and Meaney, 1993). Animals subjected to short periods of neonatal-handling show an increased ability to cope with stressful stimuli as adults, whereas prolonged periods of maternal separation results in a decreased ability to adapt to stressful events in adulthood (Anand and Scalzo, 2000; Anisman et al., 1998; Cirulli et al., 2003; Ladd et al., 2000; Lehmann and Feldon, 2000; Levine, 2002; Newport et al., 2002; Pryce and Feldon, 2003). Conflicting results appear to be due to large variations in the experimental protocols used, such as frequency and duration of separation, individual or litter separation, temperature and light:dark cycle as well as different controls used for comparisons (Lehmann and Feldon, 2000; Pryce and Feldon, 2003).

The underlying mechanisms for the effects of short periods of neonatal-handling and prolonged periods of maternal separation are believed to be mediated, in part, by maternal behaviour. Brief periods of handling does not represent a period of maternal depriva-

tion, because over the course of the day mothers are routinely off their nests and away from pups for periods of twenty to twenty-five minutes. However, upon reunion the mother shows an increased maternal behaviour of licking and grooming her pups (Liu et al., 1997; Pryce et al., 2001). During semi-naturalistic conditions, subordinate females have been shown to be forced to locate nests at some distance from food and water sources, resulting in more prolonged periods of separations (Calhoun, 1962). Such prolonged periods of maternal separation appear to alter maternal behaviour and subsequent mother-pup interactions. Upon reunion, maternally-separated dams show a decrease in the amount of licking and grooming towards the pups (Hout et al., 2000).

In summary, artificial manipulations of the dam have shown that the mother is highly responsive to subtle environmental cues resulting in a change in behaviour towards the offspring. Manipulations that alter the level of maternal licking and grooming of the young, consequently, have long-lasting effects on brain development and behavioural responses in the offspring. This suggests that the level of maternal licking and grooming early in life is crucial for normal brain development in the rat.

2.1.3. Natural Variations in Maternal Care

Strain differences in maternal behaviour in the rat have developmental consequences for the offspring (Cierpial et al., 1990; Frankova, 1985; Moore et al., 1997; Myers et al., 1989a). For example, strain differences in adult blood pressure between offspring of spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats are related to differences in maternal licking and grooming, retrieval of pups, and nursing posture exhibited by the two strains during the first three weeks postpartum (Myers et al., 1989a). Pups cross-fostered from a WKY mother to a SHR dam exhibit a phenotype similar to that of a biological offspring of a SHR mother, suggesting that the maternal behaviour of the caring dam is directly related to development of the offspring (Myers et al., 1989b). More recently, it was shown that individual differences in HPA responses to stress and levels of hippocampal GR within the offspring of Long-Evans rats were associated with the mother's frequency of maternal licking and grooming (Liu et al., 1997). Dams who exhibited high levels of licking and grooming were also found to engage in arched-back nursing more frequently than females who were low in licking and grooming behaviour (Caldji et al., 1998; Liu et al., 1997), however,

no group differences were found in overall time spent in contact with pups (Caldji et al., 1998). As adults, the female offspring of High LG-ABN dams are themselves high in maternal LG-ABN behaviour towards their pups and likewise, the offspring of Low LG-ABN mothers are Low in maternal LG-ABN behaviour towards their pups (Francis et al., 1999). Using the cross-fostering procedure, this transmission of maternal behaviour was shown not to be genetically inherited through the germ-line, but environmentally regulated by the caring mother (Francis et al., 1999). These data suggest that natural variations in maternal behaviour are inherited through a nongenomic mode of transmission.

In summary, mechanisms regulating ER-α function in the MPOA and the connections to the maternal circuit and the mesolimbic DA system appear to be crucial in regulating maternal LG-ABN behaviour towards the offspring (Numan and Callahan, 1980; Stack et al., 2002). These natural variations in maternal responses show nongenomic-inheritance, and experimental manipulations that alter levels of maternal LG-ABN change subsequent performance in terms of both physiological functions and behaviour of the offspring: adult offspring of High LG-ABN mothers show lower responses to stress in comparison to the adult offspring of Low LG-ABN mothers (Liu et al., 2000; Liu et al., 1997). Thus, the maternal brain is crucial for the transmission of maternal behaviours early in life that contribute to the development of responses to stress in the offspring that are rendered permanent through life. Stressors and the neural pathways involved in the offspring's responses to stress are described in the following section.

2.2. *The Stress Response*

Exposure to a hostile condition results in a series of co-ordinated responses organised to enhance the probability of survival. Indeed, every disturbance in the body, either real or imagined, evokes a stress response, which serves to restore homeostasis and to facilitate adaptation. The *flight* or *fight* response, also called the acute stress response, was first described by Walter Cannon in the 1920s as a theory that animals react to threats (Cannon, 1932). In 1935, Hans Selye discovered stress effects on survival unintentionally during an investigation for novel sex hormones (Rosch, 1999). According to Selye, stress is a state or condition of the body produced by diverse nocuous agents and manifested by a syndrome of changes. The agents that produced stress were termed stressors, and the syndrome,

which forms the presence of stress in the body known, was termed the general adaptation syndrome (Rosch, 1999). Thus, the *flight* or *fight* response is now recognized as the first stage of a general adaptation syndrome that regulates stress responses among vertebrates and other organisms.

2.2.1.1. Modelling Stress

Stressors can be defined as conditions that endanger, or are perceived to endanger, the survival of an individual. Such stressors can be grouped into three broad categories:

- Psychological stressors, based on a learned response to the threat of an impending adverse condition (fear, anxiety, exposure to a novel or uncontrollable environment such as 'white noise') (Campeau et al., 1991; Windle et al., 1997).

- Stressors that consist of physical stimulus and have a strong psychological component (pain, foot-shock, immobilisation, restraint) (Beaulieu et al., 1987; Beck and Rankin, 1995; Bhatnagar et al., 1998).

- Stressors which challenge cardiovascular homeostasis (haemorrhage, orthostatic stress/upright tilt, exercise, heat exposure) (Bereiter and Gann, 1986; Blair and Halperin, 1996; Bonaz and Tache, 1994; Duncan et al., 1996).

2.2.2. Stress Sensory Pathways

Sensory pathways are integral to the initial stress response. Most sensory inputs pass through either, the reticular activating system or through the thalamus, which functions as a relay station, to the sensory cortex (Amiragova, 1985). The sensory cortex then communicates this information to the perirhinal cortex, which in turn can communicate either directly or via the hippocampus with the lateral amygdala (LeDoux et al., 1990). The amygdala is a limbic structure with interconnections to the hippocampus, hypothalamus, and cortex (Aggleton, 1993). The amygdaloid complex is involved in the regulation and recognition of fear-motivated behaviour (Adolphs et al., 1995). The lateral-basal and central nuclei in the amygdala are distinct in functional involvement. The lateral-basal amygdala functions as a funnel and integrator of sensory input from the thalamus and cognitive information from the cortex and hippocampus, whereas the central nucleus is involved in

behavioural responses (Davis, 1994). The amygdala also plays a role in the neuroendocrine stress response (Beaulieu et al., 1986). The hypothalamus receives axons from cells in the amygdala and this amygdalo-hypothalamic pathway is believed to play a role in the adrenocortical response to a number of somatosensory stimuli (Feldman and Conforti, 1977). These stress responses are composed of alterations in behaviour, autonomic function, central nervous system (CNS) function and the secretion of multiple stress hormones, because of hypothalamic-pituitary-adrenal axis (HPAA) activation.

2.2.3. The Hypothalamic-Pituitary-Adrenal Axis (HPAA)

The primary CNS nucleus involved in the regulation of the pituitary-adrenal axis is the paraventricular nucleus (PVN) of the hypothalamus (Figure 3). The parvocellular region of the PVN (pPVN) is the principal source of the 41 amino acid ($\alpha\alpha$) peptide corticotrophin-releasing hormone (CRH) (Rivier et al., 1986; Vale et al., 1981). CRH and arginine-vasopressin (AVP), are the major physiological regulators of pituitary adrenocorticotroph (ACTH) secretion (Rivier et al., 1986). The CRH and AVP hypophysiotropic neurones from the dorsal-medial region of the pPVN project to the external zone of the median eminence (ME), and release CRH into a specialised capillary network. The anterior pituitary (adenohypothesis) is vascularised by hypophysial portal vessels that arise from these ME capillary beds. Within the anterior pituitary, CRH interacts with a specific guanine-protein-coupled-receptor (GPCR, termed CRH-R_1) on the corticotroph cell surface, resulting in the stimulation of the synthesis of the ACTH precursor peptide proopiomelanocortin (POMC) and the secretion of ACTH and other POMC-derived peptides, such as β-endorphin (Turnbull and Rivier, 1997). ACTH induces the secretion of steroid hormones from the adrenal gland. Members of the steroid hormone family include progestins, androgens, oestrogens and corticoids. Mineralocorticoids (MC) mainly regulate ion transport and fluid/electrolyte balance, whereas glucocorticoids (GC) regulate many metabolic activities, in addition to stress resistance. ACTH potently induces the secretion of GC from the zona fasciculata of the adrenal cortex. In humans, the major GC is cortisol, but in the rat and mouse, corticosterone is the main steroid product of the zona fasciculata. In a classical endocrine feedback manner, these steroids inhibit the synthesis and secretion of CRH and AVP within the hypothalamus and POMC-derived peptides in the pituitary, to prevent ster-

oid-induced cellular damage (De Kloet et al., 1998; Young et al., 1986).

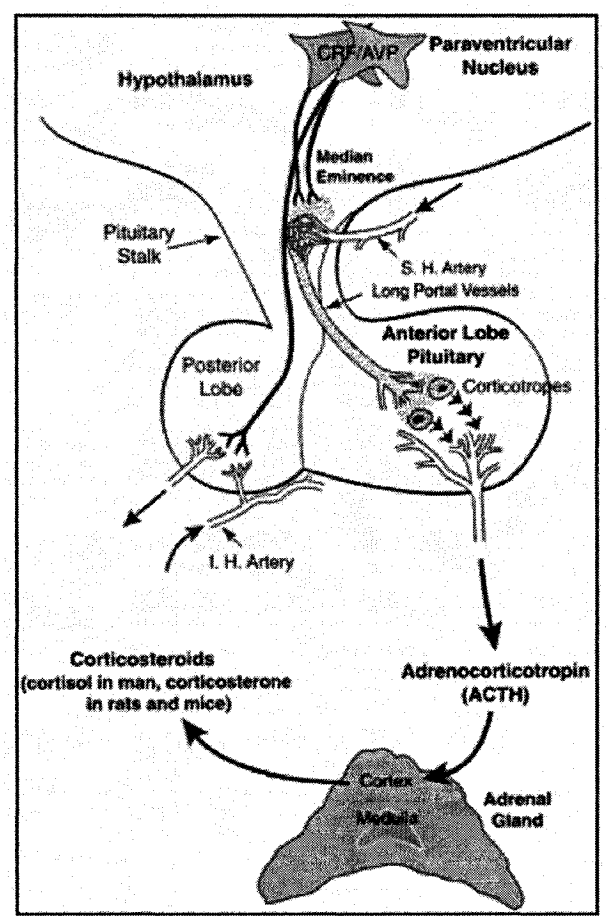

Figure 3: Functional anatomy of the hypothalamic-pituitary-adrenal axis (HPAA; see text for details). Abbreviations denote the following: AVP, arginine vasopressin; CRF, corticotrophin-releasing factor (also known as CRH); I.H, inferior hypophysial; and S.H, superior hypophysial [adapted from (Turnbull and Rivier, 1999)].

Essentially, the adrenal cortex acts as an interconnecting relay between the HPAA and sympatho-adreno-medullary system (SAMS), and contributes to the increase in cardiovascular tone and energy production, by mobilisation of energy substrates (Noback and Eisenman, 1981). The following is a closer examination of the role of the adrenal gland in steriodogenesis, with special regard to the HPAA and the sympathetic autonomic nervous system (SNS).

2.2.4. Endocrine System Stress Response

The adrenals reside extra-peritoneal at the upper poles of the kidneys and consequently

enlarge upon increased ACTH production induced by the stress response (Figure 4) (Felig et al., 1995). The glands consists of an outer cortex surrounded by a fibrous capsule and an inner medulla (Pignatelli et al., 1998). The cortex is derived from the mesoderm and consists of three distinct zones: the zona glomerulosa, which secretes aldosterone; the zona fasciculata, which secretes GC; and the zona reticularis, which secretes progesterone and androgens, mainly dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEA-S) (Felig et al., 1995; Stratakis and Chrousos, 1995). The medulla is composed of two different types of chromaffin cells arranged in epithelial cords (Karalis et al., 1997). One type of chromaffin granule has a dense core and contains noradrenaline, whilst the second type has a more defuse core and contains adrenaline (Toth and Hinson, 1995).

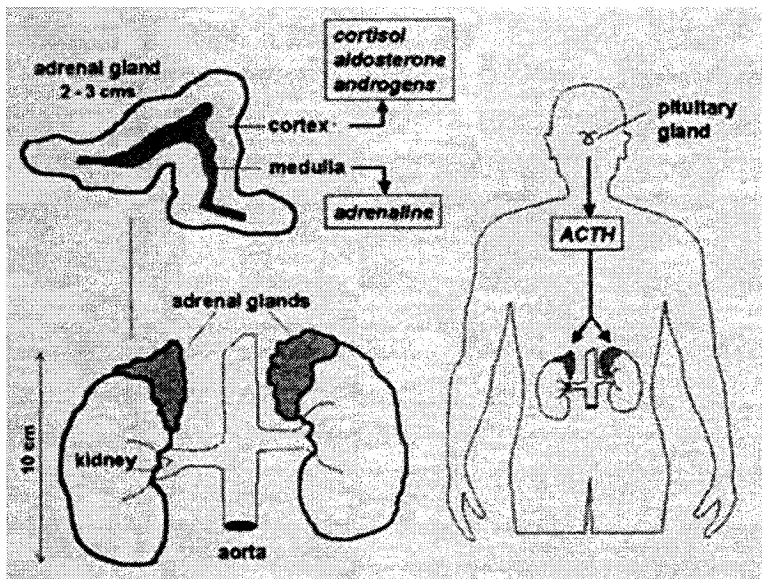

Figure 4: Functional anatomy of the adrenal gland stress-induced response (see text for details) [adapted from (Arlt and Stewart, 2005)].

During acute stress, the hypothalamus secretes CRH and AVP that act on the corticotrophs of the anterior pituitary and cause ACTH secretion. Activated sympathetic preganglionic fibres reach the adrenal gland by the splanchnic nerves, which traverse the cortex and synapse on chromaffin cells, stimulating the production of catecholamines and neuropeptides, including CRH and ACTH (Kajiwara et al., 1997). Sympathetic activity stimulates renin secretion by the juxta-glomerular apparatus in the kidneys, leading to an increase of blood angiotensin-II (A-II) levels (Kvetnansky et al., 1995). A-II is a potent vasocon-

strictor, and thus increases in levels of ACTH ultimately cause an increase in the rate of adrenal blood flow through, which facilitates the removal of secretory products, increases the supply of oxygen (O_2) and substrates to their target cells and stimulates steriodogenesis (Pignatelli et al., 1998).

2.2.4.1. Steroidogenesis: Glucocorticoid (GC) Synthesis & Release

Within the zona fasciculata of the adrenal cortex, ACTH binds to its cognate receptor (k_d = 10^{-9} M), which is coupled to a GPCR that activates adenylate cyclase (AC), which consequently leads to the activation of the cyclic-adenosine-3',5'-monophosphate (cAMP) pathway (Catalano et al., 1986). An increase in intracellular cAMP consequently results in dephosphorylation of 3-hydroxy-3-methylglutarylcoenzyme-A (HMGCoA) reductase. HMGCoA is the rate-limiting enzyme involved in the synthesis of cholesterol from dietary cholesterol transported in the plasma as low-density lipoprotein (LDL) synthesised in the liver (Brown et al., 1979). The newly formed cholesterol is then stored within the smooth endoplasmic reticulum (SER). Mitochondrial derived cytochrome P450scc mediates cleavage of the side-chain of cholesterol to form pregnanolone, which is the precursor to all steroid hormones. However, pregnanolone is further hydroxylated by P450c17 and P450c21 to yield 11-deoxycorticosterone (11-dGC), which is then hydroxylated in the mitochondria by P450c11 to form the active steroid hormone, GC. In concert, the entire process of GC synthesis and release takes between two to three minutes (Jefcoate et al., 1986).

Approximately 90-97 % of the circulating cortisol is bound by plasma proteins (Burton et al., 1971). About 90 % of this binding is with corticosteroid-binding-globulin (CBG, also termed transcortin) and to a lesser extent with albumin (Felig et al., 1995). Human transcortin is a 383 αα glycoprotein with a molecular weight of ~58 kDa, whereas rat transcortin contains a 374 αα and has a molecular weight of ~42.2 kDa (Hammond et al., 1990; Smith and Hammond, 1989). Low levels of transcortin mRNA are synthesized by extra-hepatic tissues, and these may be important for foetal development during late gestation when hepatic transcortin mRNA levels are low. Studies of the ontogeny of transcortin biosynthesis in the rat have also indicated that plasma transcortin levels may be influenced by a more rapid clearance of the protein during pubertal development. Structural organization and chromosomal location analysis shows that transcortin is a member of

the serine protease inhibitor (SERPIN) superfamily (Hammond et al., 1991). Recent studies examining the functional significance of this relationship reveal a specific interaction between transcortin and elastase on the surface of neutrophils at sites of inflammation. Transcortin is specifically cleaved, which induces a conformation change and disrupts the binding between GCs and transcortin, and promotes a significant and local release of GCs. In this context, transcortin directs the amount of GCs to sites of inflammation, such as wounds (Hammond et al., 1991).

During chronic stress, ATCH also produces a decrease in zona glomerulosa A-II receptor expression and a reduction of aldosterone synthase activity characterised by a decrease in P450scc aldosterone synthase mRNA levels in the adrenal glands (Aguilera et al., 1995). The adrenal cortex undergoes an adaptation that allows the hyper-secretion of GCs to occur without the corresponding increase of ACTH. One hypothesis is that the SNS stimulates the release of GCs before the stimulus by ACTH becomes effective (Keller-Wood et al., 1984). These high levels of ACTH lead to HPA hyper-responsivity and immuno-suppression. Essentially, the HPAA influences the immune-system through a cytokine feed-back loop involving the SNS (Toth and Hinson, 1995), and is described in the following section.

2.2.5. Stress & Illness

Historically, Louis Pasteur (1822-1895) was the first to experimentally demonstrate that stress was associated with impaired immune function. In a seminal study, he observed that chickens exposed to stress were more susceptible to anthrax than non-stressed chicken (Nicol, 1974). Ever since his late 19th century discovery, increasing amounts of evidence have supported the view that life stress is associated with a variety of diseases that vary from gastrointestinal (ulcers) to depression, cancer and increased susceptibility to infections (herpes, influenza, and common cold). The vast majority of investigations have focused on studying the effects of acute stress on the immune system, whereas the effects of chronic stress on the immune system are not as clearly defined. Thus, due to the nature of stressors that are generally associated with human pathologies (e.g., depression, infectious disease), it is clinically relevant to study the impact of chronic stress on the immune function. In addition, most studies in the stress-immunity literature are phenomenological, providing

little or no information on the underlying mechanisms of stress-related effects on immunity. Nevertheless, it is increasingly accepted that the endocrine and both the central and peripheral nervous systems regulate and can alter immune function. The most convincing example of this modulation is the activation the HPA system, which results in increased GC production by adrenals and consequently immunosuppression.

During chronic stress, the SNS stimulates the zona glomerulosa of the adrenal glands to increase the secretion of interleukin- (IL-) 1, IL-6, and tumour necrosis factor- (TNF-) α (Figure 5) (Kajiwara et al., 1997). Increased levels of IL-6 stimulate the release of CRH from the hypothalamus, thus activating the HPAA. The release of CRH from the hypothalamic pPVN consequently induces the secretion of ACTH from the pituitary, resulting in the secretion of GCs from the adrenal cortex. The secretion of both ACTH and GC is mediated by the prostaglandins and catecholamines released from the adrenal medulla (Karalis et al., 1997). Increased levels of GC-induced transcription factor activation and DNA binding-independent protein-protein interaction (cross-talk) with other transcription factors, results in the repression of AP (activator protein)-1 and NF (nuclear factor)-κB gene expression (Beato et al., 1995; Gottlicher et al., 1998; Reichardt and Schutz, 1998). These transcription factors control the expression of many immuno-regulatory genes, including cytokines, and thus their inhibition consequently results in immuno-suppression (Gottlicher et al., 1998).

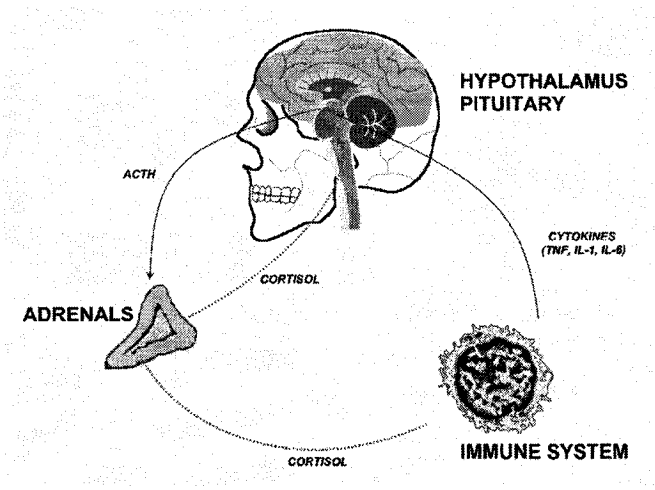

Figure 5: The functional anatomy of stress-induced immuno-suppression (see text for details). Dashed lines denote down-regulation, whereas solid lines denote activation pathways [adapted from (Bauer, 2005)].

Extended exposure to GCs have been shown to result in muscle atrophy, steroid-induced diabetes, hypertension, hyperlipidemia, hypercholesterolemia, impaired tissue growth and repair, as well as increased susceptibility to disease due to such extended immuno-suppression (Brindley and Rolland, 1989; Miller and Crapo, 1993; Munck and Holbrook, 1984). Accordingly, anxiolytic drugs of the benzodiazepine class and other therapeutics that affect catecholamine, $GABA_A$, histamine, opioid and serotonin receptors have been used to dampen the neuroendocrine stress response (Goldstein et al., 1982; Li et al., 1997). Alternatively, due to their immuno-suppressive and anti-inflammatory activity GCs are extensively used in the treatment of many medical disorders, including skin disorders, asthma, rheumatoid arthritis, inflammation, leukaemia and autoimmune diseases (Barnes, 1998). In human pathology, GC activity has been shown to be involved in erythropoiesis (Miller and Crapo, 1993). For example, patients suffering from Addison's disease have low circulating levels of GC, characterised by an autoimmune destruction of the adrenal gland and reduced cortisol production, and display severe anaemia. On the other hand, Cushing's syndrome patients show high circulating GC levels and the heamatocrit is enhanced (Miller and Crapo, 1993).

In summary, development occurs within an environmental context that ultimately shapes the function of the brain and other organs in the juvenile and adult animal. These effects ultimately define individual differences in multiple traits, including the development of behavioural and endocrine responses to stress. However, the resulting increase in stress reactivity and behavioural inhibition can serve as a risk factor for many forms of chronic illness. Herein lies the dilemma. A brief period of controllable stress may be experienced with excitement and can be beneficial to emotion and health. In contrast, lack of control and uncertainty can produce a chronic state of distress, which is believed to enhance vulnerability to disease. Thus, levels of circulating GC must be maintained under tight control. The proteins and signalling pathways involved in the GC feedback loop are described in the following section.

2.3. *Glucocorticoid Receptor (GR)-Mediated Negative Feedback System*

Basal adrenocortical activity varies throughout the day in a circadian rhythm, presumably

in anticipation of locomotor activity and food intake (Kvetnansky et al., 1995). In humans, plasma GC attains the highest levels before waking and the lowest levels before the sleeping hours. In rats, which are nocturnal, the reverse occurs (Bertini et al., 1988). In the rat, corticosterone binds with high affinity (K_d = 0.5 nM) to mineralocorticoid receptors (MR) predominantly localised in limbic hippocampal neurones and with a 10-fold lower affinity (K_d = 5.0 nM) to glucocorticoid receptors (GR) that are widely distributed within the rat brain (Arriza et al., 1987; Chao et al., 1989; Evans et al., 1985; Herman et al., 1989; Pignatelli et al., 1998; Reul and de Kloet, 1985). However, circadian peak and increased stress levels stimulate GR activation in the hippocampus (Meaney et al., 1988; Reul and de Kloet, 1985) and it is the balance in actions mediated by the two-corticosteroid receptor types within these neurones that is thought to be critical for neuronal excitability, stress responsiveness and behavioural adaptation.

Within the hippocampus, MRs are close to saturation with low basal concentrations of corticosterone, whereas high corticosterone concentrations during stress occupy both MRs and GRs (Karalis et al., 1997). Corticosterone through GR dampens the stress-induced HPAA activation in hypothalamic CRH neurones and modulates the activity of the excitatory and inhibitory neuronal inputs of these neurones. Lesions of the hippocampus or fornix increase plasma levels of GCs and ACTH and reduce diurnal variations in plasma GCs. However, though hippocampal lesions raised trough levels of GCs, peak levels of GCs remained unchanged (De Kloet and Reul, 1987). Hippocampal MRs mediate the effect of corticosterone on maintenance of basal HPAA activity and are of relevance for the sensitivity or threshold of the central stress system (Dallman et al., 1987; Jacobson and Sapolsky, 1991; Sapolsky et al., 1990). This control probably involves a steady excitatory hippocampal output, which regulates a γ-amino butyric acid- (GABA-) ergic inhibitory tone on PVN neurones. Co-localised hippocampal GRs mediate a counteracting anti-inhibitory influence. Through GR activity in ascending aminergic pathways, corticosterone potentates the effect of stressors and arousal on HPA activation (De Kloet et al., 1998; Plotsky, 1987).

2.3.1. Early Life Experience & Corticosterone Programming

GCs have potent effects on development during the perinatal period. Epidemiological evidence suggests that an adverse foetal environment permanently programs physiology, lead-

ing to increased risks of cardiovascular, metabolic and neuroendocrine disorders in adulthood (Weinstock et al., 1988; Welberg and Seckl, 2001; Welberg et al., 2001). Prenatal-stressed rat offspring have elevated plasma corticosterone (Stohr et al., 1998), increased CRH mRNA in the amygdala (Cratty et al., 1995), reduced monoamine and catecholamine turnover (Peters, 1982; Takahashi et al., 1992) and decreased MR and GR expression. These stress effects are mediated, in part, by the effects of stress on maternal behaviour of the dam (Moore and Power, 1986; Power and Moore, 1986) and the flow of GCs over the foeti-placental barrier (Welberg et al., 2000). Neonatal handling, a manipulation that increases maternal LG-ABN behaviour (see section 2.1.2), reverses the effects of prenatal stress (Wakshlak and Weinstock, 1990). The prenatal stress effects are also reversed by adoption, which results in an increase in maternal behaviour in both gestational-stressed and non-stressed dams (Maccari et al., 1995). Cross-fostering studies showed that gestational-stressed mothers spend less time maternal anogenital licking the offspring. Furthermore, non-stressed offspring fostered to gestation-stressed dams are more active [in an open-field arena (see section 4.4 for the paradigm)] than prenatal-stressed offspring reared by non-stressed dams (Moore and Power, 1986). Thus, stress during pregnancy can alter the maternal behavior of stressed dams, and the differential maternal behaviour affects emotional behaviours in the offspring that are rendered permanent through life. Importantly, maternal LG-ABN behaviour during the early postnatal period appears to be important for the reversal of prenatal stress effects in the developing neonate.

2.3.1.1. Stress Hyporesponsive Period

During the first two weeks of postnatal development, the basal secretion of corticosterone and ACTH is markedly diminished. Importantly, low circulating GC levels during the first two weeks of life are believed to be essential for normal brain and behavioural development. At this time, the response to stress is reduced and so this period is termed the stress hyporesponsive period. However, some stressors, such as insulin-induced hypoglycaemia, overcome this inactive state and stimulate ACTH release (Bertini et al., 1988). In the rat, the adrenal cortex maybe less sensitive to the stimulatory effect of ACTH during the first two weeks of life. Indeed, the reduction in the corticosterone levels is much more pronounced than the reduction in ACTH levels. The decrease in corticosterone synthesis and secretion, during this postnatal period in the rat, maybe explained by the limited stores of

adrenal cholesterol or the limited transportation of cholesterol to the mitochondria, the rate limiting factor in steroidogenesis. Indeed, the enzymes involved in adrenal steroidogenesis, including P450-scc and 3β-hydroxy-steroid dehydrogenase (3β-HSD), are markedly low in the adrenal of the developing rat (Pignatelli et al., 1998). The hypo-secretion of CRH during the neonatal period maybe the result of an increased negative feedback of GCs, due to the increased levels of free corticosterone in relation to the low levels of transcortin. However, following the stress hypo-responsive period, the plasma levels of corticosterone progressively increase to reach adult levels (Kajiwara et al., 1997).

In summary, GR function within the hippocampus appears to be the determining factor in the GC feedback system. The distribution of GR within the rat hippocampus is well established (Aronsson et al., 1988; De Kloet et al., 1998; Herman et al., 1989; Sousa et al., 1989; Van Eekelen et al., 1988; Yang et al., 1998). What follows is a brief description of the hippocampus structure, including the location of GR expression.

2.3.2. Hippocampal GR Topography

The hippocampus is a bilateral limbic structure formed from a tri-synaptic circuit with intrinsic connections between Ammon's horn [areas cornu ammonis- (CA-) 1 and CA3], the dentate gyrus, and the subiculum of the rostral septal pole, of the dorsal hippocampus (Figure 6) (Amaral and Witter, 1989; McEwen, 1997). Each hippocampi receives most afferent input from the entorhinal cortex through a fibre bundle, termed the perforant path, which forms synapses with granule cells in the molecular layer of the dentate gyrus (Amaral and Witter, 1989). The neurones of the dentate gyrus extend their axons along the CA3 stratum radiatum (mossy fibres) to pyramidal cells in area CA3 of Ammon's horn. These neurones extend their axons (Schaffer collaterals) to synapse on apical dendrites of pyramidal cells in the lacunosum-moleculaire of area CA1, thereby completing the tri-synaptic circuit (Schumacher et al., 1997).

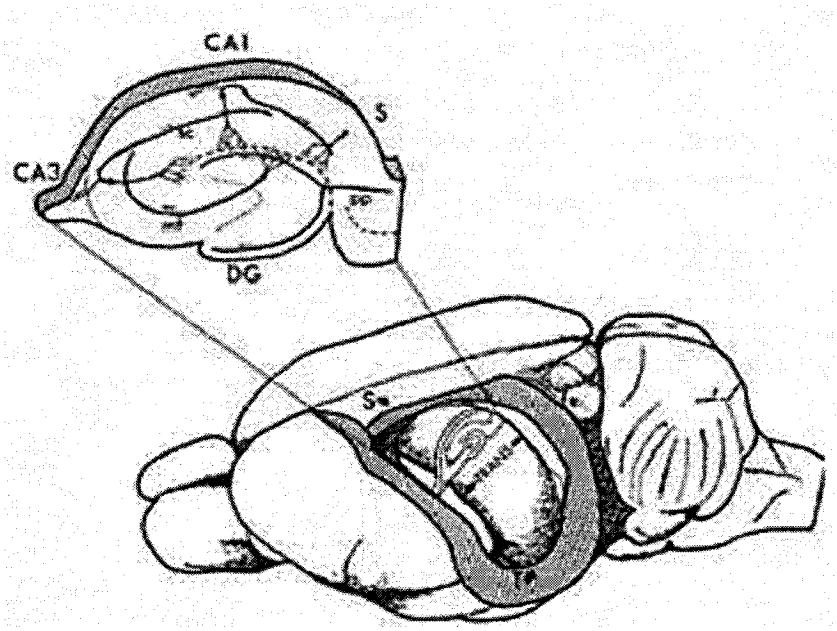

Figure 6: Location of the hippocampus in the rat brain (see text for details). Abbreviations denote the following: cornu ammonis-1, CA1; cornu ammonis-3, CA3; dentate gyrus, DG; and subiculum, S [adapted from (Fuster, 1995)].

All cells within the hippocampus formation, including the pyramidal cells of Ammon's horn, dentate gyrus cells and elements within the subiculum express high levels of GR mRNA transcripts. GR expression is highest in the CA1, subiculum and dentate gyrus, slightly lower in the CA2 and lowest in the CA3 cell field. However, this image from autoradiograph data can be somewhat misleading because it does not reflect differences in cell size or packing (i.e., cell density). For example, granule cells within the dentate gyrus are small and tightly packed, measuring approximately half the size of CA3 pyramidal neurones, where density is lower. Moreover, there is a clear gradient in cell density among the different cell populations of Ammon's horn (CA1<CA2<CA3=CA4). Therefore, the actual cellular expression of GR, when measured as grains/cell, is only moderately different within the various cell fields (Seckl et al., 1993).

However, despite similarly high cellular expression of GR in all hippocampal fields, CA1 and CA3 pyramidal neurones are by far the most vulnerable to high levels of GCs (Landfield et al., 1977; Meaney et al., 1988; Sapolsky, 1985). The potential role of cell death during neuronal development includes optimisation of synaptic connections, removal of unnecessary neurones and pattern formation. Indeed, a central feature of the brain is its

capacity to form new connections in order to compensate for injury or changes in the environment, termed neuroplasticity, and is described in more detail below.

2.3.3. Neuroplasticity & Synaptic Transmission

The process of cell formation (neurogenesis) in the hippocampus mostly terminates several weeks before birth, with the newborn hippocampus containing all cell types and cell layers characteristic of the adult hippocampus. Neurogenesis is primarily a developmental process that involves the proliferation, migration and differentiation into neurons of primordial CNS stem cells. The changes in the features of the postsynaptic target neurones of hippocampal granule cells indicate connections between granule cells and their target neurons are immature at birth. In the subgranular cell layer of the DG, hippocampal progenitors continually proliferate, migrate and differentiate into neurones in the granule cell layer of the dentate gyrus during the first six months of life (Altman and Das, 1965; Kempermann and Gage, 1999; Seress, 1998). Neuroplasticity occurs at synapses through changes in synaptic density and connectivity within the hippocampus, termed synaptogenesis (Smith et al., 2000). Synaptogenesis occurs with both proximal and distal neurones throughout life in response to environmental stimuli, most notably in response to learning (Gould et al., 1999a; Gould et al., 1999b; Ormerod and Galea, 2001). These transient changes in hippocampal synapse provide an efficient mechanism of neuroplasticity without requiring dramatic change in hippocampal morphology.

Bliss and Lomo have shown that increased synaptic efficiency can be induced in the hippocampus in response to intense repetitive stimulation [initially termed long-lasting potentiation (LLP), but now known as long-term potentiation (LTP)] (Bliss and Lomo, 1973). However, the synaptic membrane must be strongly depolarised and expressing glutamate-bound *N*-methyl-*D*-aspartate (NMDA) receptors coupled to voltage-gated Ca^{2+} channels. Following receptor activation, the influx of Ca^{2+} acts as an intracellular second messenger that initiates the local changes responsible for LTP in the dorsal hippocampus. In young adult rats, GCs increase both the amplitude and the duration of Ca^{2+}-dependent slow after-hyper-polarisation (AHP) due to an increased slow Ca^{2+}-dependent potassium (K^+) conductance (Joels and de Kloet, 1989; Kerr et al., 1991; Pfaff et al., 1971). Ca^{2+} entry through voltage-gated Ca^{2+} channels in hippocampal neurones is greater in aged rats in comparison

with young animals (Landfield and Pitler, 1984). Indeed, LTP formation is facilitated when GCs are administrated in low concentration. However, hippocampal LTP formation has been shown to decrease in a dose-dependent manner in the presence of GCs (Foy et al., 1987). Thus, a U-shaped function exists between levels GC and LTP (Diamond et al., 1992).

Attention determines the content of memory, and retrieved memories serve as the basis of expectations and direct attention. Thus, a change in memory would be expected to result in a change in attention and expectations, and vice versa. However, a decline in memory and learning is commonly observed in aged rodents and in most human neurodegenerative diseases and is largely attributed to neurone loss within the hippocampus. Basal HPA activity increases with age, resulting in elevated ACTH and corticosterone plasma levels. Chronic treatment (3 months) of young adult rats with high levels of GCs or prolonged exposure to stress produces a similar hippocampal neuropathology to that observed in the aged animals (Kerr et al., 1991; Sapolsky, 1985). In the classic model of hypoxic ischaemia, where all blood flow to the brain is transiently blocked, the presence of high GC concentrations increase the amount of hippocampal damage or accelerate the onset of damage (Koide et al., 1986; Morse and Davis, 1990; Sapolsky, 1985). These findings, taken together with the maternal effects on hippocampal GR sensitivity, suggests a relation between maternal care, GC sensitivity and neurone survival in the developing rat hippocampus.

2.3.4. Neurotrophin Support & Neurone Survival

Though development in mammals is affected profoundly by environmental stimuli, those provided by the mother are most critical for cell survival and tissue growth (Butler and Schanberg, 1977; Butler et al., 1978; Evoniuk et al., 1979; Kuhn et al., 1978; Kuhnert et al., 1979; Pauk et al., 1986). In the rat, neonatal maternal touch alters the hippocampal expression of immediate early genes (IEGs) including c-Myc and max (Wang and Kelly, 1996). c-Myc promotes cell-cycle progression and the biosynthesis of molecules necessary for normal growth (Heby and Emanuelsson, 1981; Marton, 1987; Slotkin and Bartolome, 1986). Essentially, tactile stimulation has immediate impact on endocrine function in the infant rat, including the stimulation of neurotrophic factors and growth hormones that me-

diate the development of the hippocampus (Schanberg et al., 1984). These neurotrophins stimulate cell proliferation, differentiation, axonal and dendritic arboration, and survival of specific neuronal populations (Cattaneo and McKay, 1990; Kalcheim et al., 1992; Trupp et al., 1996; Wright et al., 1992).

Brain-derived neurotrophic factor (BDNF) is expressed by neurotrophic tyrosine kinase receptor type-1 ($NTRK_1$, also termed TRKA)-positive primary sensory neurones (Apfel et al., 1996), most of which are nociceptive C fibres that mediate tactile stimulation (Michael et al., 1997; Yan et al., 1997; Zhou and Rush, 1996). In the hippocampus, BDNF produces phosphorylation of the NMDA receptor-1 (NR_1) subunit, which increases the frequency of entry of the channel into the open state (Iwasaki et al., 1998; Levine et al., 1998; Suen et al., 1997; Zirrgiebel et al., 1995). Such systems known to mediate experience-dependent neuronal development are affected by maternal behaviour, and actively stimulate hippocampal synaptogenesis in the offspring (Kirkwood et al., 1993; Schatzki et al., 1990). Furthermore, spatial-learning and memory is dependent upon hippocampal integrity (Morris et al., 1982; Squire, 1992; Whishaw, 1998; Wood et al., 1999). Indeed, an increase in peripheral levels of nerve growth factor (NGF), which is also retrogradely transported from the periphery to the cell body, would activate such signal transduction pathways that may, via CREB, contribute to increased BDNF expression and cell growth (Greene and Kaplan, 1995; Hendry et al., 1974; Tao et al., 1998). This suggests that neurones are initially overproduced and then compete for target-derived neurotrophic factors (Burek and Oppenheim, 1996; Cowan et al., 1984). Neurones out-competed for such growth factors may undergo programmed cell death, apoptosis (word origin Gk: the dropping of a petal or leaf from a flower or tree) (Kerr et al., 1972).

Although GR was originally found to mediate the orderly demise of activated immune cells when they have completed their function, apoptosis occurs throughout the nervous system in neurone, glial and neuronal progenitor cells. It has been estimated that at least half of the original cell population is eliminated as a result of apoptosis in the developing nervous system (Burek and Oppenheim, 1996; Oppenheim, 1991). This active cell death is characterised by nuclear shrinkage, chromatin condensation, cytoplasmic constriction with a reduction in cell volume, cell membrane blebbing/convolutions, extrusion of

membrane bound cytoplasmic fragments (apoptotic bodies) and DNA fragmentation. Passive cell death or necrosis, is characterised by nuclear and cytoplasmic swelling, chromatin flocculation, cell membrane and nuclear membrane dissolution or lysis evident as ghost cells (Adams and Cory, 1998; Nunez et al., 1998). Specific hormones and/or growth factors have been shown to mediate the apoptosis pathway (Ashkenazi and Dixit, 1998; Evan and Littlewood, 1998; Green and Reed, 1998; Thornberry and Lazebnik, 1998) and are described in more detail below.

2.3.4.1. Apoptosis Cascade

Apoptosis is initiated through receptor and non-receptor pathways, involving the TNF receptor family (Group I); mitochondria (Group II); and endoplasmic reticulum (ER) (Group III) (Figure 7). Importantly, both intracellular and extracellular apoptotic signals converge to activate a group of apoptotic-specific cysteine proteases, termed CASPases, which cleave their substrates with signature specificity at Asp-Xxx bonds (i.e., following aspartic acid residues) (Thornberry, 1997; Thornberry and Lazebnik, 1998). Activation of the members of the TNF receptor family results in receptor trimerization, and consequently the co-localisation of CASPase 8 and fatty acid synthase (FAS) within the receptor's cytoplasmic FAS associated death domain (FADD). FAS activity leads to cleavage of CASPase 8, which is then able to cleave and activate other members of the CASPase family, including CASPase-3, which has a high affinity for chromatin and migrates to the nucleus. Furthermore, receptor-mediated increases in intracellular Ca^{2+} levels lead to calcineurin-dependent phosphatase mediated dephosphorylation of cytosolic B-cell lymphoma (BCL)-2 associated protein-X (BAX), which is then involved in the non-receptor mediated apoptosis pathway (Adams and Cory, 1998).

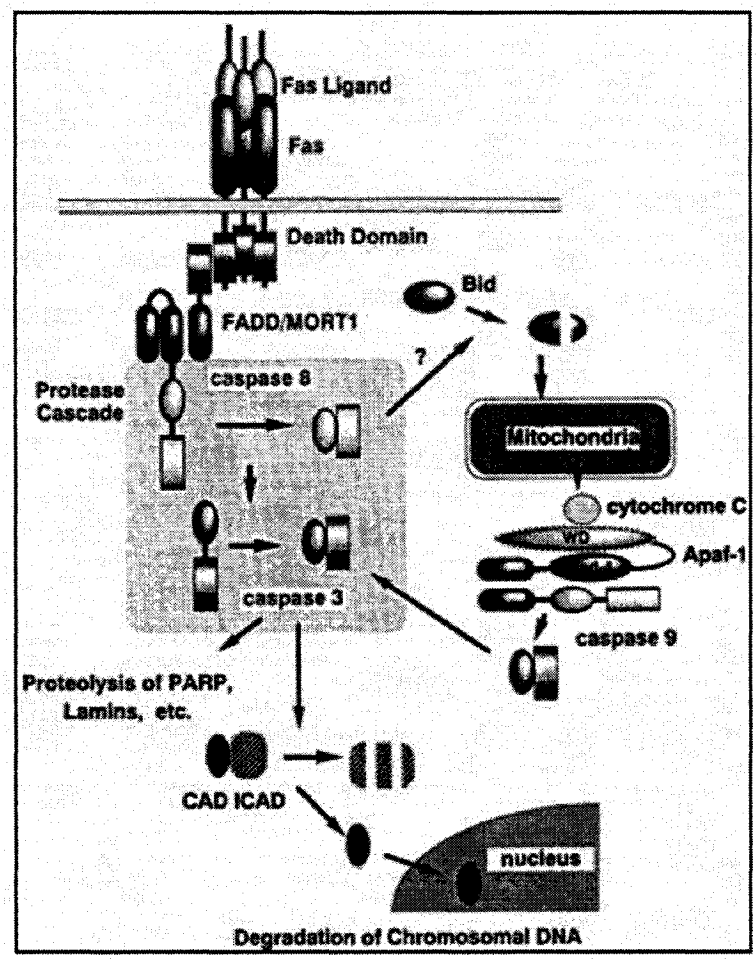

Figure 7: Flow diagram illustrating the apoptosis cascade (see text for details) [adapted from (Burek and Oppenheim, 1996)].

Non-receptor apoptosis pathways are involved in the stabilisation of mitochondrial integrity and the prevention of release of pro-apoptotic polypeptides (Korsmeyer et al., 1993). Indeed, a major regulatory step for CASPase activation is at the level of cytochrome carboxylase (Cyto-C) release from the mitochondria to the cytosol. Thus, the balance between pro- and anti-apoptotic BCL-2 family members is important for neuronal survival during development (Merry et al., 1994; Nijhawan et al., 2000). Among the genes involved in the regulation of such pathways, the BCL-2 proto-oncogene family, consisting of anti-apoptotic members [BCL-2 and BCL-x_L, BCL-W, MCL-1, A1 (Group II)] and pro-apoptotic members [BAX, BAK, BOK (Group I); and BAD, BOK, BIK, BID, BCL-2-x_s (Group III)], are best defined (Adams and Cory, 1998; Antonsson and Martinou, 2000; Gross et al., 1999; Oltvai et al., 1993). Immuno-electron microscopic studies have shown

that the BCL-2 protein localises within the mitochondria, where the inner and outer mitochondrial membranes are closely opposed, occluding the mitochondrial transition pore (MTP) (Krajewski et al., 1993). Apoptotic effectors, including both BAX (21 % homologous to BCL-2) and dephosphorylated BAD, heterodimerise with death-repressing molecules through highly conserved BCL-2 homology domains (BH1-BH4). Following heterodimerization with BCL-2, the pro-apoptotic proteins insert into the outer mitochondrial membrane, to form channels, and recruit other mitochondrial outer membrane proteins, including the voltage-dependent anion channel (VDAC) protein and the adenosine nucleotide transporter (ANT) (Muchmore et al., 1996; Reed et al., 1996; Shimizu et al., 1999). Indeed, VDAC is a subunit of the MTP and the opening of the pore results in rapid loss of membrane potential, resulting in organelle swelling, outer membrane rupture, and the release of intermembrane proteins such as apoptosis inducing factor (AIF) into the cytosol (Kroemer et al., 1997). Moreover, the outer membrane of the mitochondria becomes permeable to Cyto-C (Adams and Cory, 1998; Oltvai et al., 1993; Reed, 1997; Sattler et al., 1997).

When released from the mitochondria Cyto-C activates an apoptosome complex, formed of both pro-CASPase-9 and apoptosis activating factor-1 (APAF-1), in the presence of dATP. Cyto-C release not only initiates CASPase-3 activation by activating APAF-1, but also breaks the electron transfer chain (ETC), resulting in reduced energy generation and increased reactive O_2 species (ROS) formation due to incomplete reduction of atomic O_2 to form water (H_2O) (Reed, 1997). The activated CASPase-3 has a high affinity for chromatin and migrates to the nucleus. Consequently, the nuclear located CASPase selectively cleaves vital genomic regulatory proteins, including DNA-dependent protein kinase (DNA-PK), mRNA splicing protein (U1-70 K), sterol regulatory element binding protein (SREBP), and proteins involved in cytoskeletal re-organisation (Adams and Cory, 1998; Kumar et al., 1997). Cleavage of the DNA repair protein, poly-ADP-ribose polymerase (PARP), leads to the lysis of the nuclear DNA into 180 bp fragments, a process termed DNA 'laddering' (Oltvai et al., 1993).

Activation of the CASPase family is also associated with differences in mitochondrial phosphatidylserine (PS) expression. PS, phosphatidylethanolamine, spingomyelin and

phosphatidylcholine form the normal constituent of the mitochondrial cell membrane and are usually restricted to the inner leaflet. However, CASPases inactivate of the enzymes '*translocase*' and '*floppase*' that actively pump PS to the inner-mitochondrial membrane, and activate of the enzyme '*scramblase*' that equilibrates the membrane lipids on the inner and outer leaflet of the cell membrane. Consequently, PS is expressed on the outer leaflet of the mitochondrial cell membrane (Zwaal and Schroit, 1997). The final cellular pathology includes cytoplasm condensation and chromatin aggregation, and ultimately the neurone fragments into membrane-covered pieces expressing PS, which are then selectively targeted for digestion by phagocytosis (Allen et al., 1997).

In summary, the prodigious effects of GCs on the structure, function, and survival of hippocampal neurones are mediated, in part, through the cellular expression of GR. The steroid-bound form of GR interacts with sequence-specific genomic response elements (RE) and regulates transcriptional activity (Beato, 1987; McEwen, 1996; Rousseau, 1984). Thus, the actions of GC on GR alter the expression of cellular mRNA transcripts and proteins and influence the fundamental properties of hippocampal neurones. The functional property of the GR protein and the mechanism by which the steroid receptor regulates transcription is more fully described below.

2.3.5. Functional Motifs of the GR Protein

GR belongs to the nuclear receptor super-family of ligand-dependent transcription factors, containing over 150 members with a common primary structure and spanning a large diversity of animal species (Mangelsdorf et al., 1995). Other members include the progesterone, oestrogen, retinoic acid, thyroid hormone and vitamin D_3 receptors. Human GR (hGR) was the first hormone receptor to be cloned and sequenced (Hollenberg et al., 1985). More recently, hGR was shown to be subjected to alternative translation initiation from a downstream, in-frame ATG codon, resulting in two protein isoforms, termed hGR-α and hGR-β (Yudt and Cidlowski, 2001). Although hGR-α is a hormone-activated transcription factor (777 αα, 94 kDa), the C-termini truncated hGR-β isoform (742 αα, 91 kDa) is unable to bind hormone and therefore transcriptionally inactive. However, GR-β can heterodimerise with GR-α and inhibit hormone-activated GR mediated transcriptional transactivation

(Bamberger et al., 1996; Oakley and Cidlowski, 1993). Indeed, steroid and thyroid hormones exert their effects in patterns of gene expression by binding to specific DNA sequences in target genes and altering rates of transcription (Glass et al., 1997). The availability of cDNAs encoding theses proteins made it possible to delineate the four functional domains (Figure 8) required for ligand-binding (LBD), dimerization (hinge, H), DNA-binding (DBD) and transcriptional transactivation (Landers and Spelsberg, 1992).

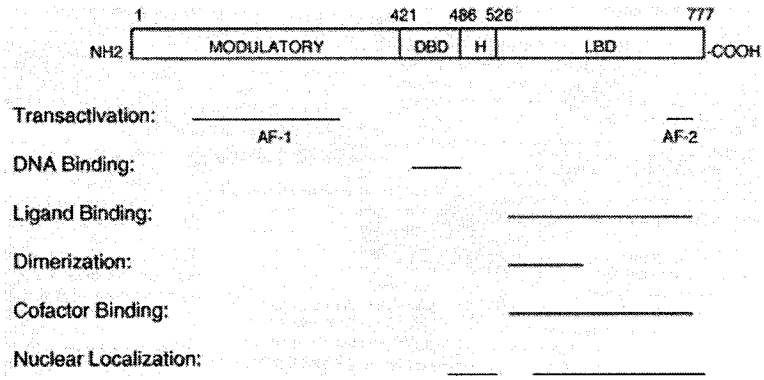

Figure 8: Functional motifs of the rat glucocorticoid receptor (rGR, see text for details). Abbreviations denote the following: DNA-binding domain, DBD; hinge, H; ligand-binding domain, LBD; constitutive activation function-1, AF1; and ligand-dependent activation function-2, AF2 [adapted from (Glass, 1994)].

The N-terminal is highly acidic, and is thought to be involved in cell-type specific regulation of gene transcription, receptor dimmer formation and receptor-transcription factor heterodimer formation (Evans, 1988; Laudet et al., 1992). The cysteine rich DBD spans the core domain (~66 αα) and confers homodimer formation and DNA binding recognition for hormone response elements (HRE) (Beato, 1991; Evans, 1988). The DBD is organised into two zinc finger modules that interact with HREs in a helix-turn-helix configuration (Berg, 1989; Klug, 1995). This core domain contains two α helices, one of which (P box/ recognition helix) engages the major groove of DNA to make specific contacts with hydrogen bonds of the bases of the HRE half-site [Arg-447 to G^2, Val-443 to T^3 (Glu-203 in ER), Lys-442 to G^{-5}] (Dahlman-Wright et al., 1991; Luisi et al., 1991; Schwabe et al., 1993). The hinge domain is composed of a short hydrophobic sequence (~40-70 αα) linking the central DBD to the C-terminal. Picard and Yamamato (1987) attached the GR hinge domain to non-nuclear proteins and found the resulting protein hybrid to be localised in the nucleus (Picard and Yamamoto, 1987), whilst mutational analysis in latter studies demon-

strate that both the hinge and DBD are vital for GR's transcriptional transactivation property (Hollenberg et al., 1985). The C-terminal represents over one-third of the entire protein (~225 αα) and is responsible for determining hormone-binding, dimerization properties, hormone-dependent transcriptional transactivation functions and hormone reversible transcriptional repression (Glass, 1994). GR studies in which single αα were either inserted or deleted resulted in loss of both steroid binding capacity and the ability to transactivate hormonally responsive genes (Danielsen et al., 1987; Giguere et al., 1986; Hollenberg et al., 1987; Rusconi and Yamamoto, 1987). Deletion of the entire LBD resulted in constitutive nuclear localisation of the truncated receptor and transcriptional activation. Implying, that the C-terminal domain is important for GR's cellular localisation (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987; Miesfeld et al., 1987). Interestingly, the C-terminus of GR is necessary for the interaction with chaperone proteins, confirming the pivotal role of this domain in maintaining the cytosolic location of the receptor (Housley et al., 1990; Picard et al., 1988; Scherrer et al., 1993).

2.3.6. Signaling Pathways of GR-Mediated Transcriptional Regulation

Due to their lipophilic nature and relatively small size, the fat-soluble steroid hormones are thought to pass through the lipid bilayer of the cell membrane by passive diffusion (Plagemann and Erbe, 1976). On the other hand, corticosteroid-selective membrane transporters maybe involved (Ballard, 1979). Unoccupied GR resides in the cytoplasm of target cells as part of a multimeric complex (~300 kDa), called the transportosome. The transportosome is composed of heat shock protein (hsp)-90, hsp70, and hsp56 (Pratt, 1992; Pratt and Welsh, 1994). These are molecular chaperones, which are important in mediating the receptor's role in binding steroid, protein folding/unfolding and nuclear transport (Bresnick et al., 1989; Hutchison et al., 1993; Pratt, 1993; Pratt and Welsh, 1994). Importantly, the interaction of hsp90 with the receptor's LBD is vital for maintaining the untransformed transportosome bound to microtubules in the cytoplasm (Akner et al., 1994; Martins et al., 1991; Picard et al., 1988; Pratt et al., 1988; Yamamoto et al., 1988). In general, entry of the hormone from vascular circulation into the cell and binding to the cytoplasmic receptor complex occurs within minutes of injection (Landers and Spelsberg, 1992).

X-ray crystallography studies suggest that cytosolic GR has a canonical structure,

consisting of twelve α-helical regions and a conserved β-turn, sandwiched between anti-parallel bundles of helices 1-5 and 6-12, which forms the hydrophobic core of the LBD (Bourguet et al., 1995; Renaud et al., 1995). Helix-12 contains the conserved AF-2 core, which projects away from the LBD in the holo-structure. Upon hormone binding, the receptor's structure becomes more compact: the loop region between helices 2 and 3 is tucked under helix-6, and consequently helix-12 becomes tightly folded against helix-4, which, in turn, makes direct contact with the bound hormone (Wagner et al., 1995). This allosteric change also involves auto-phosphorylation and consequently dissociation of hsp90 to expose nuclear localisation signals and a DBD, resulting in the activated form of the steroid receptor (Grody et al., 1982; Orti et al., 1992; Picard and Yamamoto, 1987; Pratt, 1992; Pratt et al., 1989). The activated transportosome possesses a greater affinity for chromatin and translocates to the nucleus via two possible mechanisms; either by, passive diffusion of the receptor through pores of the nuclear membrane or via active transport (Dingwall and Laskey, 1991; Newmeyer and Forbes, 1988; Silver, 1991). Under basal conditions approximately 15 % of putative GR proteins are steroid-bound, while following a 30 min period of restraint stress over 89 % of the GR proteins are occupied with concomitant GR translocation to the nucleus (from 15 % to 57 %) (Meaney et al., 1988).

Once inside the nucleus, the monomeric receptor forms a homodimer through the LBD, which undergoes a conformation change, rotating the DBD 180 ° relative to the LBD (Beato et al., 1989; Evans, 1988). The re-oriented asymmetrical head-to-tail structure is then able to bind to its cognate HRE, formed from palindromic direct repeats (DR) of a hexad core sequence separated by three nucleotides, AGAACAnnnTGTTCT (Drouin et al., 1992). GR's recognition helix (Arg-447, Val-443, Lys-442) fits into the major groove of the targeted DNA sequence. The X-ray structure of the GR homodimer reveals a polar head-to-tail assembly of the two proteins on one side of the DNA double helix, occupying adjacent major grooves (Rastinejad et al., 1995). Moreover, the C-termini of both DBDs traverse the DNA minor groove, projecting orthogonally across the axis of the double helix toward the LBDs. Once bound to DNA, GR is then capable of modulating transcription of the target gene (Chalepakis et al., 1990; Herman, 1993) (Figure 9).

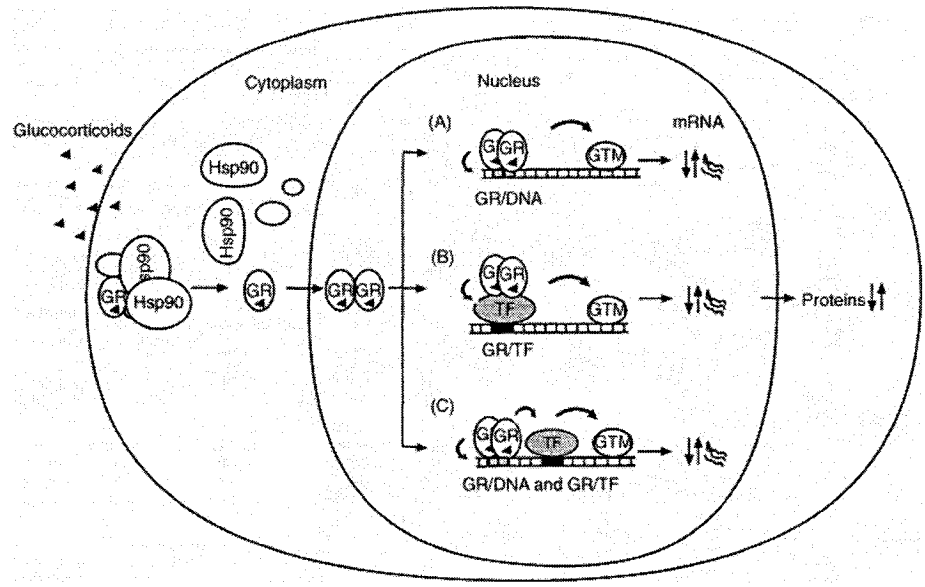

Figure 9: Signalling pathways of GR-mediated transcriptional regulation (see text for details). Following binding to GCs, the cytosolic GR dissociates from chaperone proteins such as hsp90 and translocates into the nucleus. Once there, GR readily dimerizes and modulates target gene transcription via: (A) direct interaction with cis-DNA elements including GREs and nGREs; (B) cross-talk with other DNA-bound transcription factors such as AP-1, NF-κB, Smad, and/or STAT; (C) interaction with both DNA elements and other transcription factors. The resulting modulation of target gene transcripts leads to altered protein expression. Abbreviations denote the following: general transcription machinery, GTM; other transcription factors, TF.

Nuclear translocation of the activated hormone-receptor complex and interaction with DNA transpires within 5-10 minutes following ligand-administration (Landers and Spelsberg, 1992). Changes in RNA transcript synthesis have been observed within fifteen to thirty minutes following hormone-GR homodimer-DNA interactions, whereas some late genes take two to four hours. Changes in protein synthesis and turnover take place within four to eight hours and major physiological effect of the hormone are observed twelve to twenty-four hours following administration (Landers and Spelsberg, 1992). Once the steroid-induced transcription factor has elicited its effects, the receptor complex immediately dissociates and the free form of GR migrates back to the cytoplasm (De Kloet et al., 1984). The dissociated cytoplasmic GR is then either dephosphorylated and recycled or rapidly degraded by proteases (Munck and Foley, 1976; Munck and Holbrook, 1988; Orti et al., 1989). Gene targeting has generated animal models, providing characterisation of the molecular action of neuronally expressed GR during development, and is discussed below.

2.3.7. Development of Stress Reponses in the Absence of GR Signalling

Several groups have generated mice with targeted GR deficiencies to investigate the role of GR in HPA axis regulation. Knock-out (KO) mice carrying a global deletion of GR (GR^{hypo} and GR^{null}) die shortly after birth because of impairment of critical maturation processes during development, including GC induced thymocyte apoptosis, activation of gluconeogenetic enzymes and induction of Ca^{2+} currents by GCs in the hippocampus, resulting in atelectasis of the lung, preventing analysis of the endocrine and behavioral consequences of GR deletion (Cole et al., 1995; Reichardt and Schutz, 1998; Schmid et al., 1995; Tronche et al., 1998). Transgenic mice have been produced in which antisense GR mRNA is expressed in neuronal and nonneuronal cells and leads to a 50 % decrease in GR protein (Barden et al., 1997). These mice show no differences in nonstressed, basal HPA axis activity. However, these GR mRNA antisense transgenic mice show impaired inhibition in response to the GR-specific agonist dexamethasone (DEX), in the DEX suppression test (DST) and decreased expression of CRH in the hypothalamus. When tested for behavioral measures of stress they were found to be less anxious (in the elevated plus maze) (Karanth et al., 1997). These mice provide important insights into the role of GR in HPA axis regulation; however, this mutation affects GR throughout the brain and body starting early in development, and it is unclear how developmental effects and GR signaling in areas outside of the forebrain influence these results.

The Cre-loxP system allows a gene to be knocked-out later in life, when loss of expression may not result in morbidity, and thereby allows analysis of the mechanism by which GR controls physiological processes (Reichardt et al., 2000). Cre is a prokaryotic recombinase enzyme that excises DNA fragments when flanked by two 34 bp loxP sites (Hoess and Abremski, 1985). Consequently, many different types of mutant mice have been generated, where part of the murine GR gene is modified with loxP sites and tissue specific deletion of the fragment has been induced postnatally by expression of the Cre enzyme (Cole et al., 1995; Reichardt and Schutz, 1998; Tronche et al., 1998).

To specifically examine the molecular action of GR, the Cre-LoxP system was used to generate mice with a global point mutation (A458T) in the D-loop of one of the receptor dimerization domains, impairing the DNA binding domain (DBD), whilst not affecting

GR's ability to regulate gene expression by protein-protein interaction (Reichardt and Schutz, 1998). The mutant (termed GR^{dim}) mice failed to respond to experimentally-induced anaemia, achieved by injection of the haemolytic drug (phenyl hydrazine), resulting in an increased rate of erythropoiesis (Reichardt et al., 2000). Furthermore, the GR^{dim} mice were unable to respond to low O_2 (hypoxic) conditions, whereas the wild-type (WT) mice showed an increase in the red blood cell count, haemoglobin content and the heamatocrit. Thus, although the receptor is required for stress erythropoiesis, GR is not essential for the basal regulation of erythropoiesis (Bauer et al., 1999). However, the GR^{dim} mice were able to regulate cytokine expression and suppress-inflammation in response to systemic endotoxin (lipopolysaccharide, LPS) injection, a process that is known to be mediated by the GR cross-talk with other transcription factors (Gottlicher et al., 1998). Indeed, expression of CRH in the hypothalamus remained unaffected in the GR^{dim} mice. Overall, GR^{dim} mice appear to be a useful model for improved therapeutic approaches, involving the development of GR ligands, with highly potent immuno-suppressive and anti-inflammatory activity and reduced side-effects (Vayssiere et al., 1997).

To specifically examine the role of GR in the brain, the Cre-LoxP system was used to generate central nervous system-specific GR KO ($GR^{nes/cre}$) mice (Tronche et al., 1999). These mice show a number of alterations in the HPA axis, including 10 times greater basal (morning) plasma concentration of corticosterone, increased CRH in the PVN, and decreased ACTH in the anterior pituitary. Unfortunately, GR is deleted in the PVN, a site of major negative feedback inhibition of the HPA axis, leading to symptoms reminiscent of patients with Cushing's syndrome (severe hyperadrenalism, elevated GC levels, reduced body size, osteoporosis and fat tissue displaced toward the anterior end of the body) and confounding behavioural analysis (the mutant mice are less anxious than the WT mice) (Miller and Crapo, 1993; Tronche et al., 1998). Furthermore, in the $GR^{nes/cre}$ mice, GR is deleted early in development, again, making it difficult to separate effects resulting from alterations that occur during development from those resulting from an acute requirement for GR.

To test the hypothesis that acquired primary disruption of GR action will lead to dysregulation of the HPA axis and depression like behavior, the Cre-LoxP system was used

to generate forebrain-specific GR KO (FBGRKO) mice (Boyle et al., 2005). In this system, Cre recombinase is expressed under the control of the calcium stimulated calmodulin kinase II (CaMKII) promoter. This Cre transgene is not active until 3 weeks of age (Tsien et al., 1996), and thus avoids deletion during early brain development. Deletion of forebrain GR is not complete in the FBGRKO mice until 4-6 months of age. This time course is highly relevant to the natural progression of depression in humans, which is most commonly diagnosed between the ages of 25 and 35 years (Frank and Thase, 1999). These studies demonstrated that GR action in the forebrain regulates the circuits controlling neuroendocrine and behavioral effectors that impact upon the phenotype characteristic of major depressive disorder (MDD).

In summary, these studies illustrate that GR expression is essential for the regulation of the HPAA and the stress response, as well as for the control of emotional behaviour. Indeed, hippocampal GR function is not limited to dampening the stress response, but is rather quite extensive, playing key roles in immunology, erythropoiesis, synaptogenesis, LTP formation, plasticity and protection against injury during normal CNS development. Long-term plastic changes in the brain, including those supporting memory formation, depend on permanent functional alterations in neuronal cells that require reprogramming of gene expression. We propose that physiological stimuli and the induction of activity-dependent neurotransmission through tactile stimulation may lead to these long-lasting changes in cell phenotype and function.

2.4. *Hippocampal GR Gene Induction by Tactile Stimulation*

Activity-dependent induction of GR gene expression is dependent on increases in pituitary-thyroid activity and ascending 5-HT systems (Meaney et al., 1987; Mitchell et al., 1990; Smythe et al., 1994). Indeed, maternal tactile stimulation appears to increase plasma levels of the thyroid hormone triidothyronine (T_3, liothyronine) (Meaney et al., 1996). Elevated levels of T_3 increases activity within ascending serotonergic systems, resulting in increases in 5-HT turnover in the hippocampus and frontal cortex, but not in the hypothalamus and amygdala, which are areas where neonatal-handling has been shown to have no affect on GR expression (Meaney et al., 2000; Mitchell et al., 1990; Smythe et al., 1994). Meaney and colleagues (2000) have recently characterised the cellular and molecular events that

may underlie the effect of postnatal handling on hippocampal GR gene expression (Meaney et al., 2000). Indeed, they have shown that 5-HT can directly increase hippocampal GR expression *in vivo*, and the effect is mediated by induction of the cAMP pathway and a range of cAMP-responsive transcription factors. The possible sequence of events is described below.

2.4.1. Serotonin & Hippocampal 5-HT$_7$ Receptor Activation

Within the hippocampus 5-HT has a high affinity for its cognate receptors on the surface of serotonergic cells. Many studies suggest that the serotonergic regulation of GR expression in hippocampal neurones is mediated by the 5-HT$_7$ receptor during the first week of postnatal life (Lovenberg et al., 1993a; Meaney et al., 2000; Tsou et al., 1994). However, manipulations that increase levels of LG-ABN by the rat mother have no effect on hippocampal 5-HT$_7$ receptor mRNAs levels in PND 7 offspring (Meaney et al., 2000). Molecular cloning of this family has shown that the 5-HT$_7$ receptor subtypes are members of the GPCR super family, consisting of seven hydrophobic transmembrane (7-TMD) α-helices joined by alternating intracellular and extracellular loops, an extracellular N-terminal domain, and a cytoplasmic C-terminal domain. The asparagine residues present in the extracellular domain are glycosylated for correct folding of the mature receptor peptide. In addition, a disulphide bridge is located between two highly conserved cysteine residues in the second and third extracellular domains, and two conserved cysteine residues within the C-terminal receptor domain, which have been shown to be palmitoylated. The 5-HT binding site is located within the hydrophobic-pocket delimited by TMD-I and TMD-II, approximately 15 °A distal from the extracellular surface (Lovenberg et al., 1993a; Plassat et al., 1993; Shen et al., 1993; Tsou et al., 1994).

Upon binding of 5-HT, the 5-HT$_7$ receptor undergoes a rapid conformational change and the cytoplasmic surface of the protein becomes catalytically active. The highly conserved triplet of aspartic acid, arginine, and cysteine residues (Asp-Arg-Cyst) located at the N-terminal of the i2-loop of the active receptor is critically involved in selective activation of an α_s-subunit from the Gq/G11 family, by the exchange of bound GDP for GTP. The activated α_s-subunit is then able to activate both AC type-I and AC type-II enzymes. AC activity increases formation of the second-messenger cAMP, which binds and activates

cAMP-dependent protein kinase A (PKA) (Lane-Ladd et al., 1997; Matsuoka, 1994; Sharma et al., 1980). Activated PKA rapidly undergoes a conformational change, to form a catalytic domain and four regulatory type-II subunits. The PKA catalytic domain phosphorylates the serine-133 site of a 43 kDa cAMP response element-binding protein (CREB), to form phosphorylated-CREB (pCREB) (Habener et al., 1990). In addition, the α_s-subunit of the 5-HT_7 receptor is able to regulate the activity of the β-isoforms of phospholipase-C (PLC). PLC hydrolyses phosphatidyl-inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which mediate the opening of intracellular calcium (Ca^{2+}) stores. Furthermore, 5-HT_7 receptor activation is coupled to the opening of L-type Ca^{2+} channels and the influx of Ca^{2+} into the cytoplasm. Moreover, Ca^{2+}/calmodulin-dependent protein kinase, including PKC, also phosphorylates CREB, resulting in increased levels of the transcription factor pCREB (Enslen et al., 1994; Gonzalez et al., 1989; Konradi et al., 1994; Sheng et al., 1991).

The pCREB protein binds to specific DNA sequences, termed CREB response elements (CRE), found in the promoters of many neuronally-expressed IEGs, including AP-2 and nerve-growth-factor-inducible-protein- (NGFI-) A (also know as AT225, EGR1, G0S30, KROX-24, TIS8, ZENK, ZIF-268 and ZNF225) (Habener et al., 1990; Hyman et al., 1988; Meaney et al., 2000; Milbrandt, 1988; Montminy et al., 1986; Self and Nestler, 1995; Sheng and Greenberg, 1990; Tsukada et al., 1987). Interestingly, CREB has a role in auto-regulation of the cAMP pathway, by mediating increases in AC type-VII expression (Lane-Ladd et al., 1997; Widnell et al., 1996). However, down regulation of AP-2 and NGFI-A is mediated by CRE modulators (CREMs), including the expression of inducible cAMP early repressor (ICER), which antagonise housekeeping CRE activators (Luckman and Cox, 1995; Mellstrom et al., 1993).

2.4.2. Regulation of the Immediate Early Genes AP-2/NGFI-A

IEGs provide a mechanism by which short-term cell surface events, such as tactile stimulation, are coupled to long-term changes in late onset gene expression (Beckmann and Wilce, 1997; Sheng and Greenberg, 1990). Both AP-2 and NGFI-A genes contain four distinct serum response elements (SRE) as well as several CRE-like sites (Beckmann and Wilce, 1997). Both AP-2 and NGFI-A are expressed in developing dendrites in conjunction with

stimuli that induce long-term potentiation (LTP) in granule cells of the dentate gyrus (Crino et al., 1998; Worley et al., 1993). Yau and colleagues (1996) have shown a positive correlation between NGFI-A mRNA in the CA1 region of the hippocampus and spatial learning performance (Yau et al., 1996). Importantly, both AP-2 and NGFI-A are linked to the steroid receptor super family as well as to hippocampal plasticity. These transcription factors have a zinc-finger structural motif comprised of three zinc fingers arranged in a modular C-shaped structure that allows them to fit tightly into the major groove of the DNA target sequence and can function either as an activator or suppressor of gene transcription (Beckmann and Wilce, 1997; Pavletich and Pabo, 1991). Indeed, both AP-2 and NGFI-A bind as homodimers to consensus sequences up-stream of the GR promoter and synergistically transactivate transcription of human GR (hGR) (Nobukuni et al., 1995; Wong et al., 1998).

2.4.3. Relationship Between Maternal Behaviour, 5-HT, AP-2/NGFI-A & GR Activation

In vitro, 5-HT increases NGFI-A and AP-2 expression in hippocampal neurones and the effect is blocked by the PKA inhibitor, H8 (Humblot et al., 1997; Mitchell et al., 1992). The effect of 5-HT on NGFI-A and AP-2 expression, as well as the effect on GR levels, are mimicked by the 5-HT_7 receptor agonist 5-carboxytryptamine (5-CT). These effects are blocked by either ritanserin or ketanserin, but not by pindolol; a pharmacological profile that is consistent with the 5-HT_7 receptor (Humblot et al., 1997; Lovenberg et al., 1993a; Lovenberg et al., 1993b; Plassat et al., 1993). Importantly, the effects of maternal tactile stimulation on hippocampal expression of NGFI-A and AP-2 are greatly reduced or completely eliminated in the offspring by pre-treatment with thyroid hormone synthesis inhibitors or 5-HT receptor antagonists. Thyroid hormones have been found to increase NGFI-A mRNA expression in the hippocampus, and this effect appears to be directly mediated by a thyroid receptor response element (TRE) (Ghorbel et al., 1999; Pipaon et al., 1992). The effect on hippocampal cAMP levels is blocked, either by the 5-HT receptor antagonist, ketanserin, or the thyroid hormone synthesis inhibitor, propylthiouracil (PTU) (Meaney et al., 1987). Moreover, the thyroid hormone effect occurs only during the first weeks of life, a period that corresponds precisely to the known critical period for the effect of activity-dependent expression of GR in the hippocampus (Meaney and Aitken, 1985; Mellstrom et

al., 1994). Furthermore, maternal LG has no effect on offspring's mRNA expression of NGFI-A and AP-2 in the amygdala, hypothalamus or somatosensory cortex, which are regions, unlike the hippocampus, where GR expression also remains unaltered by maternal behaviour. Finally, recent findings suggest a relationship between NGFI-A and GR expression during development. Environmental enrichment has been found to increase hippocampal expression of both NGFI-A and GR (Mohammed et al., 1993; Sarrieau et al., 1988). Taken together these findings provide a possible mechanism for maternal programming of hippocampal GR gene expression (summarized in Figure 10).

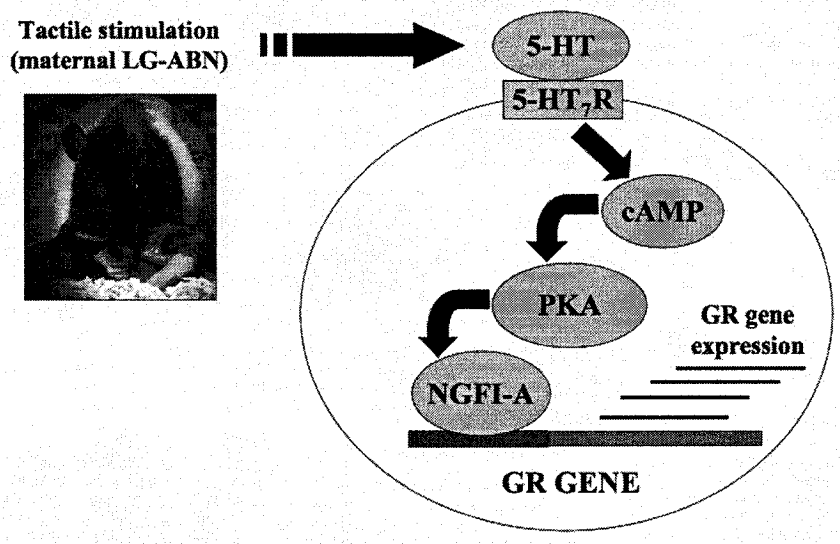

Figure 10: Maternal programming of hippocampal GR gene expression (see text for details). Maternal LG-ABN of the offspring increases hippocampal 5-HT turnover and activation of a 5-HT_7 receptor, which is positively coupled to cAMP. Increased cAMP activity results in activation of PKA and CREB. Subsequent pCREB activity drives expression of the transcription factor NGFI-A, which, when bound to the GR promoter (shaded dark grey) may drive the observed increase in hippocampal GR expression in the offspring.

Across species, increasing complexity is associated not so much with an increase in the number of genes that actively code for proteins, but rather with the size of the non-coding region of the genome. The increased size of the regulatory region of the genome should correspond to an increased capacity for environmental regulation of gene expression, a process whereby an increasing range of phenotypes might emerge from a common

genotype: an increased capacity for phenotypic plasticity. The main challenge of a complex organism, such as the rat, is how to regulate the expression of a certain set of genes from a repertoire of genomic sequences. Hippocampal GR expression, like all genes, is controlled by the epigenome, which is comprised of chromatin structure (Kadonaga, 1998) and DNA methylation (Razin, 1998) and is described in the following section.

2.5. *Epigenetic Regulation of Hippocampal GR expression*

The phenotype of the cell is a function of the genes that it expresses, which is determined by the nuclear packaging of DNA (Figure 11). Chromatin assembly is required to package newly synthesised DNA into chromatin following cell division. However, in non-dividing (post-mitotic) cells, such as neurones, chromatin assembly maintains the integrity of the genome during histone turnover (Kadonaga, 1998). The basic repeating structural unit of chromatin is the nucleosome. Both X-ray and electron crystallographic analysis reveal that a nucleosome comprises of an average of 150 bp of DNA wrapped around the nucleosomal core of histones in 1.75 turns of left-handed superhelical DNA (Kornberg, 1974).

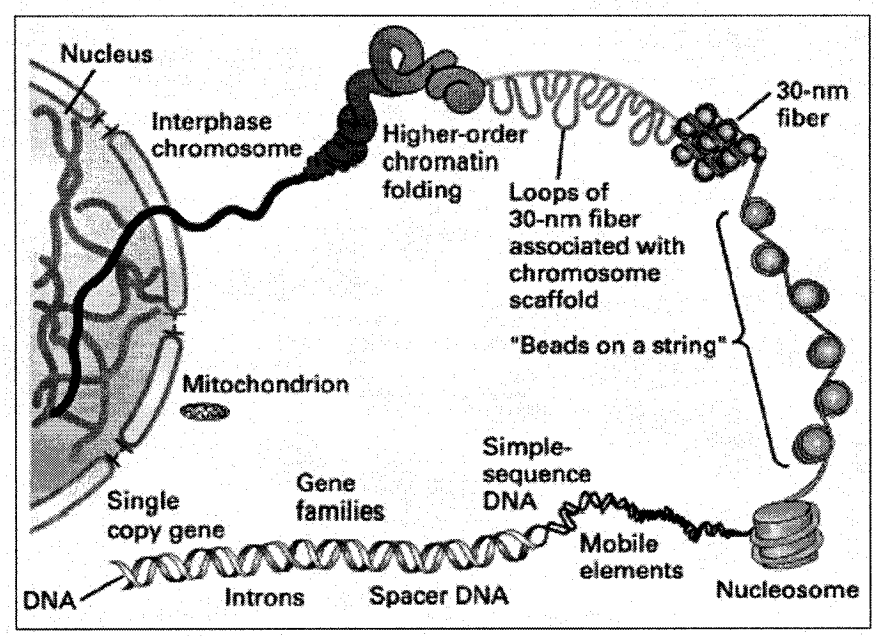

Figure 11: The nuclear organisation of the genomic DNA sequences (see text for details).

The histone-DNA configuration is maintained by electrostatic bonds between positively-charged histones and negatively-charged DNA, and regulates gene expression

(Grunstein, 1997). Five classes of histones have been identified in chromatin: histone H1, H2A, H2B, H3 and H4. Each nucleosome contains two H2As, two H2Bs and two H4s in the core. Histone H1 occurs in chromatin in about half the amount of the other types of histones and appears to lie on the outer portion of the nucleosome. Histones of the nucleosomal core can be acetylated and deacetylated, and the amount of acetylation is controlled by the opposing activities of two types of enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs). Substrates of these enzymes include ε-amino (NH_2) groups of lysine (K) residues located in the amino-terminal tails of the histones. When HDAC removes the acetyl group from histone lysine, a positive charge is restored to the lysine residue condensing the structure of nucleosomes (Davie and Chadee, 1998). Nucleosomes containing highly charged hypoacetylated histones bind tightly to the phosphate backbone of DNA, inhibiting transcription, presumably, because transcription factors, regulatory complexes and RNA polymerase do not have access to the DNA. Indeed, this closed chromatin structure commonly precludes transcription-factor binding to DNA and underscores the importance of enzymes that modify histone-DNA interactions. On the other hand, HATs catalyze the acetylation of selected positively charged amino acids (e.g., lysine and arginine) on the protruding histone tails, most commonly histone (H-) 3 or H4. In particular, histone acetylation of K9 residues on the ε-NH_2 tails of histones H3 neutralises the positive charge of the histone tail and decreases its affinity to negatively charged DNA and generates a more open DNA conformation resulting in breathing of the 146 bp DNA wrapped 1.65 turns around the octomer of histone residues (Hong et al., 1993; Sealy and Chalkley, 1978). Transcription factors and the transcription apparatus then have access to the DNA, and expression of the corresponding genes is facilitated. Thus, H3-K9 acetylation is a marker of active gene transcription (Figure 12).

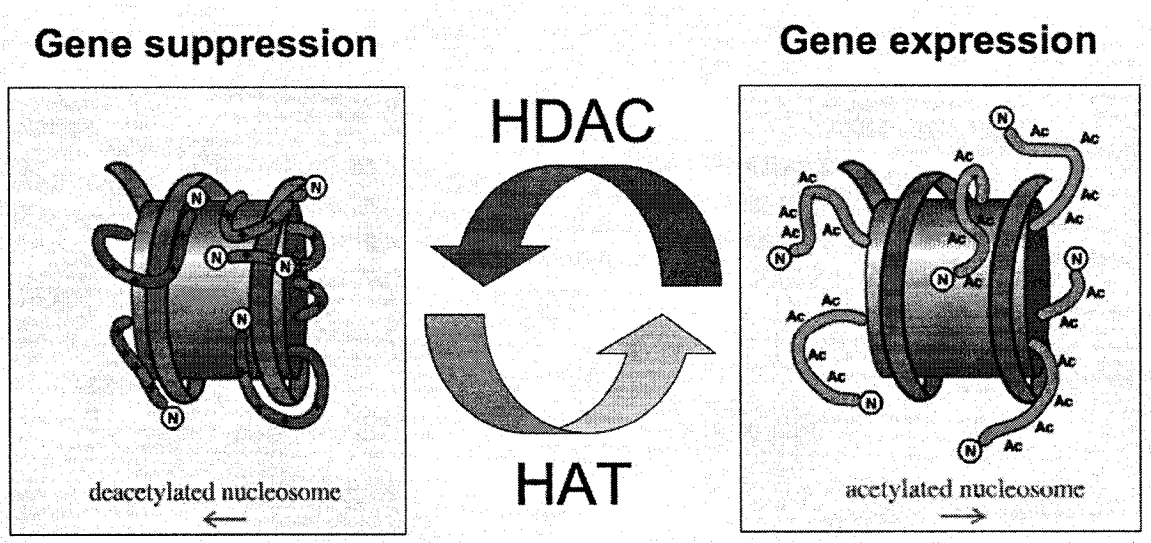

Figure 12: Regulation of histones of the nucleosomal core by the opposing activities of histone deacetylases (HDACs) and histone acetyl transferases (HATs). Chromatin fractions enriched in actively transcribed genes are also enriched in highly acetylated core histones, whereas silent genes are associated with nucleosomes with a low level of acetylation (see text for details).

Histone acetylation is regulated by HDACs, which block histone acetylation and suppress gene expression. Thus, intracellular signals can regulate gene expression through effects on HATs or HDACs (or on factors involved in other histone modifications, such as methylation, phosphorylation, ribosylation and ubiquitination) that alter chromatin structure (Bird, 2001; Bird and Wolffe, 1999; Li, 2002; Razin and Szyf, 1984). Mammalian HDAC1 and HDAC2 are also associated with the Sin3 complex that includes NCo-R, SMRT and several other, yet, unidentified proteins and appear to repress gene expression by deacetylating core histones. Indeed, some environmentally regulated alterations of histone acetylation in specific promoter sequences following seizures (Huang et al., 2002; Tsankova et al., 2004) or learning (Guan et al., 2002) are likely to be caused by neurotransmitter activation of multiple signalling pathways (Crosio et al., 2003). However, such histone modifications are transient and cannot directly explain enduring early environmental programming effects. In addition to chromatin status, the genomic DNA is covalently modified by methylation. Chromatin structure and DNA methylation patterns are unique to each type of cell. Active chromatin is associated with hypomethylated DNA, whereas inactive chromatin is associated with hypermethylated DNA.

2.5.1. DNA Methylation

The term DNA methylation is used to describe a post-replicative modification, in which a DNA residue acquires a covalently bound methyl-group (Laird and Jaenisch, 1996). A hallmark of the vertebrate genome is that modification of cytosines by methylation occurs only when the cytosine is juxtaposed 5′ to a guanosine residue. In humans, 60-80 % of the cytosines in palindromic 5′-CpG-3′ dinucleotide sequences are methylated at the carbon-5 position (Razin and Szyf, 1984). According to (Gardiner-Garden and Frommer, 1987), CpG islands are classified as genomic sequences of > 200 nucleotides in length, with a cytosine and guanosine content > 50 %, and an observed CpG over expected CpG ratio > 0.6. However, (Takai and Jones, 2003) define CpG islands as genomic sequences of > 500 nucleotides in length, with a cytosine and guanosine content > 55 %, and an observed CpG over expected CpG ratio > 0.65. In general CpG islands are approximately 1 kb in length and are usually found at the 5′ end of genes, and around 29,000 such regions exist in the human genome (Antequera and Bird, 1993; Bird, 1996; Gardiner-Garden and Frommer, 1987). The transfer of a methyl-group from the methyl donor S-adenosyl-methionine (SAM) to form 5-methylcytosine, is catalysed by the enzyme DNA methyltransferase (DNA MeTase) (Adams et al., 1979), and is described below.

2.5.2. Regulation of DNA Methylation

In mammals, only one DNA MeTase enzyme has been cloned and has a preference for hemi-methylated DNA, termed DNA MeTase- (DNMT-) 1 (Bestor and Verdine, 1994; Leonhardt and Bestor, 1993; Smith, 1994). DNMT-1 is involved in maintenance methylation of the nascent DNA strand following replication, restoring hemi-methylated CpG dinucleotides to a full double-stranded methylated state (Bestor, 1988; Bestor, 1992). Whereas, DNMT-3a and DNMT-3b are involved in *de novo* methylation, actively methylating unmethylated CpG dinucleotides within non-dividing cells (Figure 13).

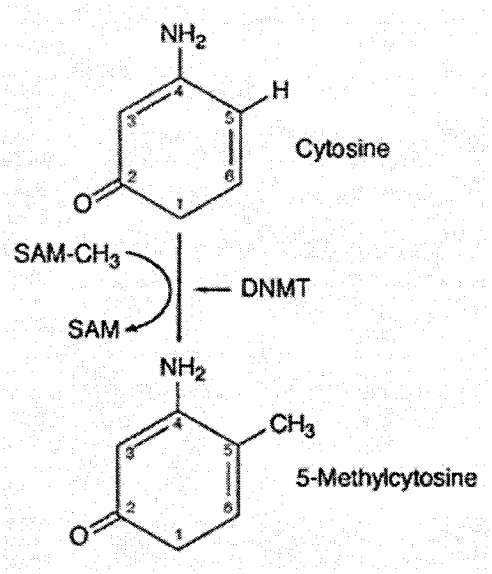

Figure 13: Mechanism of DNA methylation. 5-Methylcytosine is produced by the actions of the DNA methyltransferases (DNMT 1, 3a or 3b), which catalyse the transfer of a methyl group (CH_3) from S-adenosyl-methionine (SAM) to the carbon-5 position of cytosine.

The carbon-carbon bond between the methyl group and cytosine residue is a stable, enduring epigenetic mark. DNA methylation patterns are commonly thought to be established during early embryonic development and then faithfully maintained through life by DNA methyltransferases (Bird, 2001; Bird and Wolffe, 1999; Li, 2002; Razin and Cedar, 1977; Reik et al., 2001). The only way methyl residues were thought to be lost was through passive demethylation (i.e., replication in the absence of DNA methyltransferase) and such processes were not considered applicable to post-mitotic cells, including neurons. DNA methylation is involved in embryonic development (Brandeis et al., 1993; Kafri et al., 1992; Kafri et al., 1993; Razin and Cedar, 1993; Razin and Kafri, 1994), parental imprinting (Sapienza, 1990) and X-chromosome inactivation (Gartler and Riggs, 1983; Pfeifer et al., 1990). Mice lacking a functional gene encoding the maintenance MeTase or the *de novo* MeTase die at mid-digestion (8-somite stage) or within four weeks of postnatal life (Li et al., 1992). This raises the questions of whether cellular programming through alterations in DNA methylation is unique to this period, and whether variations in methylation marks occur solely through passive demethylation.

DNA methylation promotes gene silencing through effects on chromatin structure (Bird, 2001; Bird and Wolffe, 1999; Hashimshony et al., 2003; Kadonaga, 1998; Li, 2002;

Nan et al., 1998). In one silencing mechanism, DNA methylation may directly repel transcription factors that are unable to bind DNA when 5-mC residues occupy their recognition sites (Watt and Molloy, 1988). Indeed, many transcription factors, including AP-2, have been shown to be sensitive to 5-mC in their cognate response element (Tate and Bird, 1993). Essentially, the methylated cytosine serves as a mutation of the recognition element. However, *cis*-acting DNA elements containing Sp1 binding sites can block methylation (Brandeis et al., 1994; Macleod et al., 1994; Mummaneni et al., 1993). A second silencing mechanism is indirect, and links DNA methylation to inactive chromatin structure (Bird, 2001; Bird and Wolffe, 1999; Hashimshony et al., 2003; Kadonaga, 1998; Li, 2002; Nan et al., 1998). Whilst almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has been associated with transcriptional inactivation and formation of condensed chromatin structures enriched in hypo-acetylated histones (Eden et al., 1998; Jones and Laird, 1999). A region of methylated DNA attracts different members of a family of methylated DNA-binding proteins, such as methyl-CpG-binding protein (MeCP)-1, MeCP-2, MBD-1, MBD-2, MBD-3 and MBD-4 assemble on methylated DNA. In particular, MECP-2 associates with the histone deacetylase complex mSin3A that in turn recruits histone deacetylase (HDAC)-1 and HDAC-2, blocking histone acetylation and inactivating chromatin (Jones et al., 1998; Ng et al., 1999; Wade et al., 1999; Zhang et al., 1999). MeCP2 also facilitates chromatin inactivation through chromatin looping (Horike et al., 2005) and recruitment of Brahma (Brm), a catalytic component of the SWI/SNF-related chromatin-remodelling complex (Bachman et al., 2003; Harikrishnan et al., 2005). In addition, interactions between MBD2 and the histone deacetylase Mi-2/NuRD complex can direct the deacetylase complex to methylated DNA (Ayer, 1999). However, Bhattacharya and colleagues (1999) identified MBD2 as a DNA demethylase, suggesting that this protein might actually mediate activation of transcription rather than repression (Bhattacharya et al., 1999).

In summary, whereas the direct gene silencing mechanism refers to a discrete methylation pattern, the indirect mechanism depends on the general density of methylated cytosine residues. The model positioning DNA methylation as driving chromatin inactivation is well established. Never the less, current data suggest that chromatin structure can also determine DNA methylation, and that chromatin can affect DNA methylation in both direc-

tions, triggering either de novo DNA methylation (Cervoni et al., 2002) or demethylation (Cervoni and Szyf, 2001; Detich et al., 2003; Szyf et al., 2004). These findings indicate that DNA methylation, although extremely stable, can be altered later in life when there is a sufficiently stable and consistent signal for chromatin activation. This relationship between chromatin state and DNA methylation forms a molecular link through which environmental signals might alter DNA methylation in specific genes in post-mitotic neurons. According to this model, environmental signals trigger cellular signalling pathways, which activate trans-acting factors that recruit HATs, resulting in histone acetylation, chromatin opening and increased accessibility to DNA demethylating agents. This mechanism would enable reversal of the methylation mark by a similar intense change in chromatin structure later in life (Szyf, 2001). Thus, mapping of methylation patterns in CpG islands has become an important tool for understanding both normal and pathological gene expression events throughout life.

2.5.3. Targets for Epigentic Regulation of the Hippcampal Rat GR Gene

Linkage, somatic cell hybrids, fluorescent *in situ* hybridization (FISH) and radiation hybrid analysis have confirmed that the rat GR (rGR) gene locus (symbol Grl) is located on the short arm of chromosome 18p12 (Gauguier et al., 1996; Goldner-Sauve et al., 1991; Hilbert et al., 1991; Jacob et al., 1995; Kovacs et al., 1997; Pravenec et al., 1996; Szpirer et al., 1998; Watanabe et al., 1999). Comparison of GR cDNAs isolated from human, rat, and mouse reveal a high degree of homology between the species (Danielsen et al., 1986; Hollenberg et al., 1985; Miesfeld et al., 1986). However, the hGR contains 10 exons and has a minimum size of 80 kb, whereas the mouse GR (mGR) contains 11 exons and spans 110 kb (Encio and Detera-Wadleigh, 1991; Strahle et al., 1992). Moreover, though both hGR and mGR are formed from nine exons, there are significant differences in initiation of transcription and transcript splicing.

Only one promoter has been identified for hGR, whilst three promoters direct mGR expression. In the hGR, exons 1 and 2 encode the 5′-untranslated sequence and the N-termini, respectively. Exons 3 and 4 encode the two zinc-like fingers, whilst five exons combine to form the LBD. Furthermore, exon 9 is alternatively spliced to form two protein isoforms, α (700 αα) and β (742 αα), which differ by their C-termini (Encio and Detera-

Wadleigh, 1991; Hollenberg et al., 1985). In the mGR, the 5′-noncoding exons 1A (restricted to T-cells), 1B, and 1C (homologous to hGR exon 1 cDNA) are alternatively spliced to form a common second exon encoding for the N-termini (Strahle et al., 1992).

The rGR possesses 11 putative promoters (McCormick et al., 2000) The 5′-flanking region of the rGR is rich in CpG islands and lacks any obvious TAAT or CAAT elements, common to promoter regions of 'housekeeping genes'. The absence of TATA boxes is consistent with the presence of multiple transcription start sites, which is associated with tissue- and/or developmental-specific transcription (Schibler and Sierra, 1987). McCormick and colleagues (2000) have more recently demonstrated the tissue specific-specific 5′-heterogeneity of rGR mRNA and presented evidence for early-life environmental programming of specific promoters in the hippocampus (Figure 14).

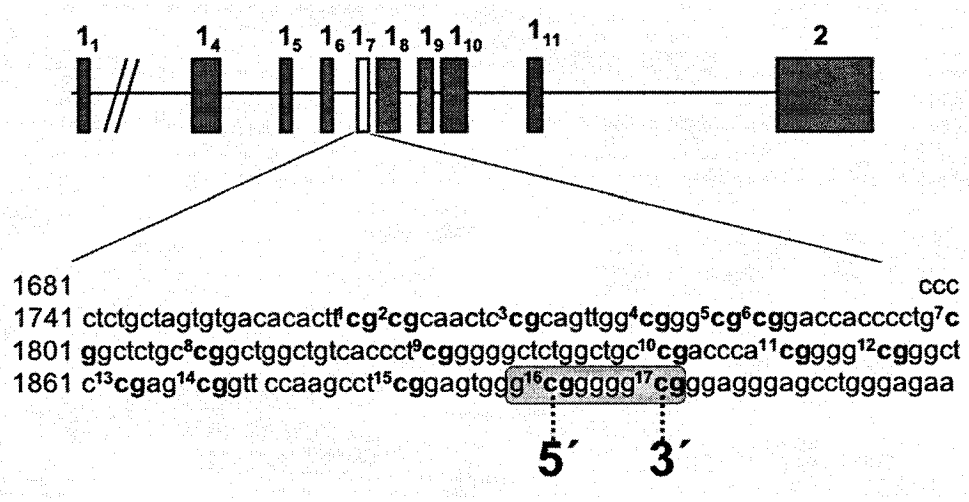

Figure 14: Sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (bold) and the NGFI-A consensus sequence (encircled) [adapted from (McCormick et al., 2000)].

The 3 kb 5′-flanking region of the rGR gene was shown to encode at least 11 alternate exons; with exon 1_1 positioned approximately 15 kb upstream of exon 2. However, only six of the remaining eight alternate exon-1s have been detected in rGR mRNA *in vivo*. None of the alternate exon 1 sequences are likely to alter the αα sequence of the GR protein, because there is an in-frame stop codon present immediately 5′ to the translation initiation site in exon 2 common in all the mRNA variants. In the adult rats, GR predominantly encoded exon 1_{10} transcripts, whilst exon 1_6 expression, also ubiquitous, was in substantial

minority. Exon 1_1 containing GR mRNA was expressed in a tissue-specific manner within the thymus, with expression in both thymocytes and thymic epithelium, whereas 1_7 expression was unique to the brain. In addition, exon 1_7 promoter constructs fused to luciferase within exon 1_7 had the highest activity. Consistently, GR mRNA transcripts containing exons 1_7 showed significant expression in the hippocampus *in vivo*. RNase protection assays have shown exon 1_7 mRNA to be present in approximately 10 % of the total GR mRNA normally expressed in the hippocampus. Furthermore, only the exon 1_7 variant GR mRNA was induced in the hippocampus by handling, with a ~three fold increase across all hippocampal fields. Implying, that neonatal handling programs increases hippocampal GR expression through increased transcription from a novel promoter upstream of the 1_7 exon.

The 4600 bp sequence of the rGR gene flanking the 5'-end of exon 2 contains the exon 1 sequence (-3269 to -3322, 1_6) and corresponds to a CpG island (68 % CG, with a CG/GC ratio of $\geq$ 0.8 between -1620 and -4520 relative to the transcription start at +1, within exon 2). Interestingly, the rGR sequence shares 91 % homology with mGR, throughout the whole region, and ~70 % homology with hGR, over the CpG island (Cole et al., 1992; Govindan et al., 1991; Zong et al., 1990). Within the CpG island, the GR gene contains 16 GC boxes (GGGCGG), which may form the consensus Sp1 site and may also bind NGFI-A (Crosby et al., 1991; Kadonaga and Tjian, 1986). Importantly, immediately upstream of exon 1_7 there is a sequence exactly matching the consensus binding-site for the family of zinc finger proteins that includes NGFI-A.

In summary, maternal care can regulate the expression of genes in the brain that influence emotional and cognitive development. One of the primary targets for the effect of maternal care is on hippocampal GR expression. The exon 1_7 GR promoter sequence contains consensus sequences for NGFI-A binding, which may be involved in mediating these environmental effects on synaptic development. Differences in methylation of specific CpG dinucleotide sequences may account for differences in gene expression throughout life. This would support the hypothesis that alterations in DNA methylation are required for differential regulation of gene expression. In particular, the effects of maternal behaviour maybe associated with changes in specific proteins induced by LG-ABN and that the permanence of this effect may be due to differential methylation of DNA sites and specific

protein-DNA interactions during the first week of life. The elucidation of the mechanisms involved in the effect of maternal behaviour addresses perhaps the most challenging question in development: How are effects occurring in early life rendered permanent? Obviously, in the case of sustained changes in gene expression in brain cells, we can begin to imagine the neurobiological basis for the development of individual differences in personality and cognition.

Chapter 3
Objectives

I hypothesise that a facet of maternal behaviour (i.e., licking and grooming) in the rat affects hippocampal development and HPA responses to stress in the offspring and that these effects are rendered permanent throughout life by epigenomic programming of the exon 1_7 GR promoter sequence. To test this hypothesis, I used an animal model that has previously been shown to express differences in hippocampal GR gene activity and HPA stress responses as a function of maternal care. The specific objectives are the following:

- To evaluate whether maternal cares effects hippocampal expression of cell survival markers in the adult offspring.
- To establish whether maternal care effects epigenomic programming of exon 1_7 GR promoter sequence and HPA responses to stress in the adult offspring.
- To establish whether the effects of maternal care on hippocampal GR gene regulation and HPA responses to stress are reversible in the adulthood.
- To examine the global effects of maternal care and reversibility on the hippocampal transcriptome in the adult offspring.
- To investigate the mechanism by which developmental programming of the exon 1_7 GR promoter sequence occurs.

Chapter 4
General Material & Methods

Although animal models provide powerful tools for behaviour and physiology, it is difficult to evaluate molecular and cellular processes directly *in vivo*. Therefore, in addition to observing animal behaviour, I evaluated cellular responses by means of *in vitro* assays that mimic the functioning of stimulated neuronal cells. Thus, a comprehensive study of epigenomic programming of stress responses was achieved by using both observational and explorative methods.

4.1. *Maternal observations*

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Constant, Québec). The animals were mated with males drawn randomly from a breeding stock maintained in our colony. In cases where the offspring of High or Low LG-ABN mothers were used in studies, no more than two animals per group were drawn from any single mother. At the time of weaning on day 22 of life, pups were housed in same-sex, same litter groups of 3-4 animals per cage until day 45 of life, and two animals per cage from this point until the time of testing which occurred no earlier than 100 days of age. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. Behavioural observations were performed with mothers and their litters housed in 46 x 18 x 30 cm Plexiglas cages that permitted a clear view of all activity within the cage. Food and water were provided *ad libitum*. The colony was maintained on a 12:12 light: dark schedule with lights on at 0800h. Maternal behaviour was scored using a version of the procedure described by Myers *et al.* (Champagne et al., 2003; Myers et al., 1989a). The behaviour of each dam

was observed for six, 100-minute observation periods daily for the first ten days postpartum. Observations occurred at regular times each day with four periods during the light phase (0900, 1200, 1500 and 1800) and two periods during the dark phase of the L: D cycle (2100 and 0600h). Within each observation period, the behaviour of each mother was scored every three minutes (25 observations/period x 6 periods per day = 150 observations/mother/day). All observations were performed by individuals unaware of the origin of the animals. On occasion, due to disturbances in the animal room, observation sessions were uncompleted. While all mothers were observed for exactly the same number periods, there was some variation across days. The data were therefore analyzed as the percentage of observations in which animals engaged in the target behaviour. The following behaviours were scored: mother off pups, mother licking/grooming any pup, mother nursing pups in either an arched-back posture, a 'blanket' posture in which the mother lays over the pups, or a passive posture in which the mother is lying either on her back or side while the pups nurse. A detailed description of these behaviours is provided in Myers *et al.* (Myers et al., 1989b). Note that behavioural categories are not mutually exclusive. For, example, licking/grooming most often occurred while the mother was nursing the pups (Stern, 1997). The frequency of maternal licking/grooming and arched-back nursing across a large number of mothers is normally and not bi-modally distributed (Champagne et al., 2003). Hence, the High and Low LG-ABN mothers represent two ends of a continuum, rather than two distinct populations. In order to define these populations for the current study we observed the maternal behaviour in a cohort of 32 mothers and devised the group mean and standard deviation for each behaviour over the first 10 days of life. High LG-ABN mothers were defined as females whose frequency scores for both licking/grooming and arched-back nursing were greater than one SD above the mean. Low LG-ABN mothers were defined as females whose frequency scores for both licking/grooming and arched-back nursing were greater than one SD below the mean. Previous reports (Levine, 1994; Meaney, 2001) of licking/grooming behaviour suggest that the frequency of licking/grooming and arched-back nursing are highly correlated ($r > +0.90$). The pups remain with the dam until weaning (PND 21), upon which the offspring were re-housed with a same-sex conspecific from then on.

4.2. *Cross-fostering*

Procedures for the adoption study were adapted from earlier studies (McCarty and Lee, 1996). Female offspring of High or Low LG-ABN dams were mated and allowed to give birth. Within twelve hours of birth, dams were removed from the home cage and two animals/litter were cross-fostered. The cross-fostered pups, along with two native pups, were labelled with a permanent marker (Codman pens from Johnson & Johnson) until day-ten of life and by individual differences in their pelage thereafter. All litters were culled to twelve pups. Subsequent studies showed that marking the pups had no effect on maternal licking/grooming; marked pups are licked/groomed no more or less frequently than unmarked pups (Champagne et al., 2003). Once the foster pups were introduced into the new litter the dam was returned. The entire procedure took less than fifteen minutes. Maternal behaviour was observed for the following hour to ensure that cannibalization did not occur. The critical groups of interest are animals born to Low LG-ABN mothers, and fostered to High LG-ABN mothers (i.e., Low: High) and the High: Low complementary group. To control for the effects of cross-fostering to another mother, the offspring of high or Low LG-ABN dams were fostered to other High or Low LG-ABN mothers, respectively (i.e., High: High and Low: Low LG-ABN groups). We also controlled for the simple effects of handling, one group of animals was fostered back onto their own mothers (i.e., a sham, cross-fostered group) as well as a group of High and Low LG-ABN mothers and litters were left undisturbed. These additional control conditions did not differ from the High: High or Low: Low within controls described above and was therefore omitted for the sake of the present experiments. Maternal behaviour of each dam was then observed (as previously described above) for the following eight days. The results confirm significantly greater licking/grooming in high compared with Low LG-ABN mothers regardless of the cross-fostering manipulation.

4.3. *Intracerebroventricular cannulae placement & infusions*

For intracerebroventricular (ICV) cannulae placement adult (PND 90) male offspring were anesthetized [Sommotol (sodium pentobarbital), MTC Pharmaceutics Ltd, Mississauga, Ontario, Canada] and the area between the ears (across the neck and scapulae) was shaven. The shaven skin was swabbed with iodine and the animal was mounted and secured in a

stereotaxic frame. A cut was made directly down the midline, and the fascia and muscle were separated to expose the skull surface, which was cleaned and dried with sterile gauze and cotton swabs. A stainless-steel guide cannula (22 gauge), 8 mm length (Plastic One Inc., USA) was aimed at the left lateral ventricle (1.5 mm posterior to bregma, 2.0 mm lateral to midline, and 3.0 mm ventral to the brain surface). Guide cannulae were secured with dental cement and three stainless-steel jeweller's screws and kept patent with stainless-steel stylets (30 gauge). The animals were placed under heat lamps until they regained consciousness and then they were transferred to single cages. Following a seven-day recovery period, animals received a single infusion every-day for seven-consecutive-days as described below. Animals were removed from their cages and gently held while an infusion cannula (28-gauge) attached to tubing (polyethylene 20), was lowered into the guide. A total volume of 2 μl of agent or vehicle was infused using a Hamilton (10 ml) micro-syringe through the infusion cannula over a 1-minute period. Infusion cannulae were left in place for an additional minute after infusion. Animals were then returned to their home cage.

4.4. *Open-field paradigm*

Open-field behavioural testing was performed as previously described (Caldji et al., 1998) using adult (PND 90) male offspring. The naive animal was removed from the home cage and placed directly into one corner of the open-field (120 cm x 120cm). The sides of the arena were black and the beige tiled floor divided into a grid of 8 x 8 squares. Movement of the animal in the arena during the 10-minute testing session was recorded by a video camera mounted on a tripod adjacent to the field. After 10-minutes, the animal was removed and returned to the home cage and the open-field arena was cleaned to prevent olfactory cues from affecting the behaviour of subsequently tested rats. All animal testing was conducted under standard lighting conditions. An observer blind to the experimental conditions coded the videotapes using a DOS based program. Exploration was defined as the time spent in the inner 6 x 6 squares, whereas overall activity was defined as the number of squares crossed during the testing session.

4.5. *Forced-swim paradigm*

Behavioural stress response testing was performed, as previously described (Porsolt et al., 1977), using adult (PND 90) male offspring. Naive animals were placed in a plexiglass cylinder (46 cm high x 20 cm diameter) filled with water (25 ± 1 °C) to a depth of 30 cm for 15-min. Following the 15-min training session, the animals were removed from the water and allowed to dry (15 min, 32 °C) before being returned to their home cages. After 24-h, the animals were replaced in the water-filled cylinder and the total duration of immobility was recorded during a 5-min test session. Immobile behaviour was scored whenever the animal remained passively afloat without struggling, in a slightly hunched but upright position with the head slightly immersed (i.e., the rat makes only those movements necessary to keep its head above the water). An observer blind to the experimental conditions scored the behaviour. The single 15-min training exposure was sufficient to produce a relatively constant level of immobility in the subsequent 5-min test, which was highly reproducible between different groups.

4.6. *Analysis of the HPA stress response*

Stress testing involved placing adult (PND 90) male offspring into Plexiglas restrainers (8.5 x 21.5 cm; Kent Scientific, Litchfield, CT) for a 20-minute period. Pre-stress blood samples were taken from rats within 30 seconds of removal from the cage, and restraint stress was performed during the light cycle between 12:00 P.M. and 3:00 P.M. with blood sampling (300 µl) from the tail vein at 10, 20, 40, 60, and 90 minutes after the onset of restraint (Meaney et al., 1989). Plasma (10 µl) corticosterone was measured by radio-immuno-assay (RIA) with a highly specific B antiserum (B3-163; Endocrine Sciences, Tarzana, CA) and [^{3}H]corticosterone (101 Ci/mmol; NEN, Boston, MA) tracer. The antiserum cross-reacts slightly with deoxy-corticosterone (~ 4 %) but not with aldosterone, cortisol and progesterone (< 1 %). The intra-assay and inter-assay coefficients of variation were 8.8 % and 10.4 %, respectively. The standard curve 50 % effective concentration was 16 µg/dl, and the detection limit of the assay was 0.63 µg/dl.

4.7. *DAPI staining and TUNEL labelling*

In the preparation of tissue for immunohistochemistry, whole brains were removed from

adult (PND 90) male offspring by rapid decapitation less than 1-min following their removal from the home cage. Coronal sections (16 μn) corresponding to stereotaxic levels from -2.30 to -3.80 mm from bregma (Paxinos and Watson, 1996) were thaw-mounted (3 sections per slide) onto gelatin-subbed slides and temporarily stored within the cryostat (-20 °C). Once sectioning was complete, the slides were vacuum-dried within a desacator (4 °C, 14 h) and stored at -80 °C. All sections were processed in parallel for terminal deoxynucleotidyl transerferase (Tdt)-mediated dUTP-biotin nick-end labelling (TUNEL) with the ApopTag Red *In Situ* Apoptosis Detection kit (Intergen Co.) following the manufacturer's instructions. Sections were immersion-fixed (10 min, 22 °C) in para-formaldehyde (1 %) in phosphate-buffered saline (PBS; 50 mM; pH 7.4), followed by two 5-min washes with PBS. Sections were then permeabilized (5 min, -20 °C) in a pre-cooled solution of ethanol: acetic acid (2:1; v/v), followed by two 5-min washes with PBS. Intergen proprietary equilibration buffer was applied to the sections (5 min, 22 °C), followed by incubation with the DNA strand break labelling solution containing Tdt enzyme (37 °C, 1 h). Negative control sections for each rat were produced by omitting the addition of Tdt enzyme. Next, sections were washed in Stop/Wash buffer (10 min, 22 °C), followed by three 1-min washes with PBS. Sections were then incubated in a solution containing antidigoxigenin conjugated to rhodamine (30 min, 22 °C), while being shielded from light. Following four 2-min washes with PBS, the sections were counterstained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (0.125 mg/ml) and allowed to dry (14 h, 4 °C). DAPI dye binds externally to DNA. The sections were then cover-slipped with Krystalon (Harleco) and visualization of TUNEL and DAPI labelling was performed on an Olympus BH2 fluorescence microscope (400x magnification). Separate filters were used to inspect the rhodamine signal [excitation wave length (ex.) 540 nm and emission wave length (em.) 550 nm] and DAPI counter-stain (ex. 365 nm and em. 480 nm). The absolute numbers of TUNEL-positive cells were counted at the same stereotaxic level over the entire area of the dentate gyrus, and CA1, CA2 and CA3 hippocampal regions of Ammon's horn. An observer blind to the experimental conditions performed the counts.

4.8. *NeuN staining and 5-mC labelling*

Preparation of tissue for immunohistochemistry as described in section 4.7. All sections

were processed in parallel. Sections were fixed (10 min, 22 °C) in paraformaldehyde (4 %), washed with 50% ethanol (30 min), blocked in 10% FCS + PBS containing 0.2 % Triton-X (2 h) and immersed in 2N HCl (2 h, 37 °C). Anti-5-mC antibody (a kind gift from Dr. Alain Niveleau, Université Joseph Fourier de Grenoble, France) diluted 1:500 in 5% serum + PBS and mouse anti-NeuN Alexa fluor 488 conjugated monoclonal antibody (Cat#MAB377X, Chemicon International) diluted 1:1000 in 5 % serum + PBS were applied to each section, which were then incubated (1 h, 37 °C) and left to hybridize (40 h, 4°C). Negative control sections for each rat were produced by omitting the addition of the primary antibodies. Rabbit anti-mouse IgG Alexa-568 (Cat#A-11061, Molecular Probes) diluted 1:600 in 5 % serum + PBS was applied to the 5-mC labelled sections. The sections were then dried (2 h) and cover-slipped with Aqua Polymount (Polysciences). Visualization of 5-mC and NeuN labelling was performed using a Zeiss Axioplan 2 Imaging fluorescence microscope (Zeiss Canada), equipped with a high-resolution colour digital camera and connected to a computer running Zeiss Axiovision 4.1 Software (Zeiss Canada). The appropriate filter combination and a 63 plan-apochromatic oil-immersion objective was used to capture images of the 5-mC-positive nuclei over the entire area of the dentate gyrus, and CA1, CA2 and CA3 hippocampal regions of Ammon's horn. These images were converted to Tiff format and imported into an MCID Elite image analysis system (Imaging Research, St. Catherine's, ON, Canada) for quantification. An observer blind to the experimental conditions performed the analysis.

4.9. *Western blot analysis*

Whole brains were extracted (as described in section 4.7) and the hippocampal tissue was dissected, snap-frozen on dry ice and stored at -80 °C. The hippocampal tissue whole-cell extract was prepared using tissue sonicated on ice (20 sec pulse at 40 °C) in TEDGEM (100 ml per 50 g of hippocampal tissue) containing NaCl (0.4 M) and protease inhibitors [aprotinin (3.5 mg), 4-(2-aminoethyl) benzenesulfonylfluouride (0.4 mg/ml), leupeptin (1 mg/ml) and pepstatin 1 mg/ml)]. Aliquots of the supernatant were subsequently taken to determine the levels of protein in the whole hippocampus. Western blots were performed using equal quantities of protein (40 μg) separated on Novex 4-12% Tris-glycine polyacrylamide gel electrophoresis (PAGE) precast gels (Helixx Technologies) with stained

molecular markers (SeeBlue; Invitrogen) loaded for reference. Proteins were then electrophoretically transferred according to the methods of Towbin (Towbin et al., 1979) onto nitrocellulose membranes (Amersham). The membranes were blocked (1 h, 22 °C) with Carnation dried milk (5 %) in Tris-buffered saline with Tween (TBS-T) [Tris, NaCl, Tween-20 (0.1 %), pH 7.6], washed briefly in TBS-T and incubated (14 h, 4 °C) with anti-rat GR-α monoclonal primary antibodies in blocking buffer (1: 4000; Affinity Bio-Reagents). Membranes were washed with TBS-T (20 min, 22 °C), and then incubated (1 h, 22 °C) with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G antibody (1: 3000; Amersham Pharmacia Biotech). Following four 15-min washes with TBS-T, the bands were visualized by enhanced chemiluminescence (ECL; Amersham) and apposed to autoradiography film (Hyperfilm-MP; Amersham) before being developed. A single band was observed at ~ 92 kDa. To verify the accuracy of sample loading, selected blots were incubated (30 min, 70 °C) in stripping solution [Tris-HCl (62.5 mM), β-mercaptoethanol (100 mM), SDS (2%)] before being blocked (14 h, 4 °C) and reprobed (1 h, 22 °C) with an α-tubulin monoclonal antibody (1: 5000; Biodesign International). A single band was observed at ~ 60 kDa and the intensity of the signal were similar in all lanes. Relative optical density (ROD) readings for the GR-α band was determined using a computer-assisted densitometry program (MCID 4.0; Imaging Research) from samples run in triplicate on three different blots. For all studies, single blots were derived from samples from one animal.

4.10. *Chromatin immunoprecipitation (ChIP) analysis*

In the preparation of fixed tissue animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, I.P.) and then transcardially-perfused with heparinized saline flush (30-60 ml), followed by para-formaldehyde (4 %) in phosphate buffer saline (pH 7.4) for 15 min. After perfusion, all brains were removed and post-fixed in the same fixation solution overnight at 4 °C and then transferred to phosphate buffer sucrose (20 %) for 48 h. ChIP assays (Crane-Robinson et al., 1999) were performed following the ChIP assay kit protocol® (Cat#06-599, Upstate Biotechnology). Hippocampi were dissected from each rat brain and chromatin was immunoprecipitated using, rabbit polyclonal anti-acetyl-histone H3 antibody (Cat#06-599, Upstate cell signalling solutions), rabbit polyclonal anti-NGFI-A antibody

(Cat#sc-189, Santa Cruz Biotechnology) or normal rabbit IgG non-immune antibody (Cat#sc-2027, Santa Cruz Biotechnology). One-tenth of the lysate was kept to quantify the amount of DNA present in different samples before immunoprecipitation (Input). Protein-DNA complexes were uncrosslinked, by adding 20 µl NaCl (5 M) to each sample (4 h, 65 °C), followed by 10 µl EDTA (0.5 M), 20 µl Tris-HCl (1 M, pH 6.5) and 2 µl PK enzyme (10 mg/ml) (1 h, 45 °C). Following phenol-chloroform (0.5 v/v) extraction, the free-DNA was ethanol (2 v/v, 95 %) precipitated with 5 µl tRNA (10 mg/ml) and re-suspended in 100 µl 1xTE. The rat hippocampal exon 1_7 GR promoter region (GenBank™ accession number: AJ271870) of the uncrosslinked DNA was subjected to PCR amplification (Forward primer: 1750-TGTGACACACTTCGCGCA-1767; Reverse primer: 1943-GGAGGGAAACCGAGTTTC-1926). PCR reactions were performed following the 'Fail-Safe PCR system' protocol using 'FailSafe PCR 2x PreMix D' (Epicentre, InterScience). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (1 min, 56 °C) and extension (1 min, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. In order to control for equal loading of acetyl-histone H3-K9 immunoprecipitate, the rat hippocampal β-actin promoter-α region (GenBank™ accession number: V01217) of the uncrosslinked DNA was subjected to PCR amplification (Forward primer: 10-TCAACTCACTTCTCTCTACT-29; Reverse primer: 161-GCAAGGCTTTAACGGAAAAT-180). PCR reactions were performed following the 'FailSafe PCR system' protocol using 'FailSafe PCR 2x PreMix L' (Epicentre®, InterScience) with the same thermocycler protocol as previously described. In order to control for purity of the NGFI-A immunoprecipitate, the rat hippocampal exon 1b oestrogen receptor (ER)-α promoter region (GenBank™ accession number: X98236) of the uncrosslinked DNA was subjected to PCR amplification (Forward primer: 1836-GAAGAAACTCCCCTCAGCAT-1855; Reverse primer: 2346-GAAATCAAAACACCGATCCT-2327). PCR reactions were performed following the 'FailSafe PCR system' protocol using 'FailSafe PCR 2x PreMix A' (Epicentre®, Inter-Science). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (1 min, 60 °C), and extension (1 min, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. PCR reac-

tions on DNA purified from non-immunoprecipitated samples and immunoprecipitated samples were repeated exhaustively using varying amounts of template to ensure that results were within the linear range of the PCR. 10 µl of the amplified products were separated on a 2 % agarose gel and post-stained with ethidium bromide (EtBr) to visualize bands corresponding to the exon 1_7 glucocorticoid receptor promoter (194 bp), β-actin promoter-α (171 bp) or exon 1b oestrogen receptor-α promoter (493 bp) DNA fragments. Nucleic acids were transferred by Southern blot (14 h, 22 °C) to positively charged nylon transfer membrane (Hybond-N^+, Amersham). An oligonucleotide (20 bp) specific for the exon 1_7 GR promoter sequence (GenBank™ accession number: AJ271870) was synthesized (Forward: 1881-TCCCGAGCGGTTCCAAGCCT-1907), as well as an oligonucleotide (21 bp) specific for the β-actin promoter-α sequence (GenBank™ accession number: V01217; Forward: 95-GTAAAAAAATGCTGCACTGTC-115) and an oligonucleotide (20 bp) specific for the exon 1b ER-α promoter sequence (GenBank™ accession number: X98236; Forward: 1942-AGAAAGCACTGGACATTTCT-1961). The oligonucleotides were radio-labelled [1 µl T4 polynucleotide kinase (PNK), Promega] with 5 µl ^{32}P γ-ATP (Amersham) (2h, 37 °C). The membranes were pre-hybridized (6x SSC, 1 % SDS, 5x Denhardt's, 0.05 % tetra-sodium pyrophosphate, 0.01 % herring sperm) (2 h, 42 °C). The radio-labelled oligonucleotides were boiled (5 min) before hybridization with the blotted membrane (14 h, 42 °C). Following two successive 10 min washes (6x SSC, 1 % SDS, 0.05 % tetra-sodium pyrophosphate) at 22 °C and four 10 min washes at 55 °C, the membranes were then subjected to autoradiography. Relative optical density (ROD) readings were determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catherine's, Ontario, Canada). To calculate the final signal for each sample, the ROD value of the band within the antibody lane (A) was divided by the ROD value of the band within the input lane (I). To control for equal loading between samples, the final signal of the exon 1_7 GR promoter, amplified from the acetyl-histone H3-K9 immunoprecipitations, was divided by the final signal from the β-actin promoter-α amplified from the same precipitate. The full protocol for this procedure, including chemical reagents and solutions, is described in section 12.2.2.

4.11. *Electrophoresis mobility shift assay (EMSA)*

NGFI-A EMSA was performed using a NGFI-A cDNA (Milbrandt, 1987) in a CMVneo expression/shuffle vector (pJOM464, Invitrogen), provided as a kind gift from Prof. Milbrandt. The NGFI-A coding sequence was sub-cloned into a TOPO-His vector (pCR®T7/CT-TOPO®, Invitrogen) following the manufactures guide (pCR®T7 TOPO® TA Expression Kits, Invitrogen). Primers (Forward: 365-GACCATGGACAACTACCCCAAA-384, Ta 66 °C; Reverse: 2553-GCAAATTTCAATTGTCCTAGG-2532, Ta 58 °C) were designed (GenBank™ accession number: M18416). The thermocycler protocol involved an initial denaturation cycle (3 min, 98 °C), 35 cycles of denaturation (30 sec, 98 °C), annealing (30 sec, 55-65 °C) and extension (1 min 30 sec, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. The ligated plasmid vector containing the NGFI-A coding region was transformed into TOP10F' E.coli cells. Colonies were selected and analyzed for insert and correct orientation by sequencing (data not shown). Bacterial cells [BL21(DE3)] transfected with the NGFI-A expression vector were allowed to grow (14 h, 37 °C), and an aliquot was removed and grown in LB ampicillin (100 mg/ml) until the OD600 of the solution reached 0.6 (3 h, 37 °C). Isopropylthio-β-D-galactoside (IPTG, Invitrogen life sciences) was added to the bacteria to a final concentration of 1 mM IPTG, and incubated further (6 h, 37 °C). The bacteria were subsequently centrifuged (4,000 rpm, 25 min, 4 °C) and the pelleted bacteria were resuspended in 10 ml lyses buffer containing one protease inhibitor cocktail tablet (Complete, Mini, Roche). The bacteria were freeze-thawed three times, sonicated (Vibra Cell, Sonics & Materials Inc., Fisher) on ice (10 sec pulse at 40 % every 20 sec for 15 min), centrifuged (10,000 rpm, 25 min, 4 °C) and the supernatant containing the recombinant protein was extracted. The recombinant protein was purified by metal-ion affinity chromatography (Schmitt et al., 1993). In brief, 1 ml of glass-wool was packed into a 3 ml syringe and washed through with 1 vol Iminodiacetic acid immobilized on sepharose 6β:fast flow elution: epoxy (IAA, Sigma). The beads were packed with 6 vol of ddH_2O and labelled with 3 vol $NiCl_2$ (200 mM). The column was equilibrated with 5 vol of ddH_2O, until no NiCl2 was eluted. The medium containing the bacteria (10 ml) was loaded onto the column. The medium that flowed through the column was collected and reloaded onto the column four times. The column was washed with 25 ml lyses buffer [Tris HCL (20

mM), NaCl (50 mM), Tween (0.05 %), Glycine (10 %)] and then again, with 10 mM and 20 mM imidazole in lyses buffer (25 ml of each) to remove non-specifically bound proteins. Elution was carried out with 250 mM imidazole in lyses buffer (4 ml) that was subsequently resuspended in 5 x binding buffer [glycine (20 %), $MgCl_2$ (5 mM), EDTA (2.5 mM), DTT (2.5 mM), NaCl (250 mM), TrisCl (50 mM, pH 7.6)] to a final volume of 15 ml. The elution containing the protein was filtered (Amicon, Ultra-15 Centrifugal Ultracel™ Low Binding Regenerated Cellulose Filter, Millipore) over four 15 min centrifugations (4,000 rpm, 4 °C), re-suspending the protein in 5 x binding buffer (to a final volume of 15 ml) between spins. Total protein was recovered following a final extended centrifugation (4,000 rpm, 25 min, 4 °C). Aliquots were taken to determine the levels of total protein. Differentially methylated oligonucleotide sequences (27 bp) of the NGFI-A consensus binding site (GenBank™ accession number: AJ271870) were used: a) non-methylated (1881-GAGCTGGGCGGGGGCGGGAGGGAGCCT-1907); b) methylated in the 5′ CpG dinucleotide (1881-GAGCTGGG**M**CGGGGGCGGGAGGGAGCCT-1907); c) methylated in the 3′ CpG dinucleotide (1881-GAGCTGGGCGGGGG**M**CGGGAGGGAGCCT-1907); or d) methylated in both CpG dinucleotide sites (1881-GAGCTGGG**M**CGGGGG**M**CGGGAGGGAGCCT-1907). The ssDNA oligonucleotides were denatured (10 min, 100 °C) with NaCl (150 mM) and Tris (pH 7.5) and annealed (3 h, 22 °C) to form dsDNA (500 mg/25 µl). The oligonucleotides were end labelled using a fill-in reaction with a DNA polymerase (Pol)-I Klenow fragment and [γ-^{32P}]dCTP. In brief, oligonucleotides were radiolabelled [1 ml T4 polynucleotide kinase (PNK), Promega] with 2 µl 32P γ-ATP (Amersham) (1h, 37 °C). The 32P γ-ATP labelled dsDNA oligonucleotide was dissolved in loading buffer [TrisCl (250 mM, pH 7.6), glycine (40 %)] and separated on a (5 %) non-denaturing acrylamide gel (200 v, 2 h, 4 °C). Labelled dsDNA oligonucleotides were eluted (14 h, 37 °C) from the gel in 1xTNE [EDTA (5 mM), Tris (15 mM, pH 7.6), NaCl (180 mM)]. Following standard phenol-chloroform DNA extraction methodology, the oligonucleotides were centrifuged (13,200 rpm, 5 min) and further precipitated [95 % Ethanol (2 v/v), 10 % Na acetate (1/10 v/v), tRNA (10 ng/ml)]. The pelleted ^{32}P γ-ATP labelled dsDNA oligonucleotide was resuspended in ddH_2O (50,000 cpm/µl). The binding reaction was conducted by incubating the end-labelled oligonucleotide probes (50,000 cpm) with purified NGFI-A protein (36 µM) and of Poly [d(I-C)] (1 µg) in 5xbinding buffer in a

final volume of 50 µl (30 min, 22 °C). For competition experiments, a 100-10,000 fold molar excess of competitor DNA is incubated in the mixture before adding the purified protein. DNA-protein complexes were resolved on a non-denaturing (5 %) poly-acrylamide gel in 0.5xTBE buffer (200 v, 2 h, 4 °C). Gels were dried and subjected to autoradiography.

4.12. *Affymetrix microarray analysis*

Whole brains were removed (as described in section 4.7) from adult (PND 90) male off-spring and the hippocampal tissue was dissected, snap-frozen on dry ice and stored at -80 °C. Total hippocampal RNA was extracted using a RiboPure™ RNA Isolation Kit (Cat#1924, Ambion), beginning at step B of the manufacturers introduction manual. The precipitated RNA was dissolved in RNase-Free H_2O and quantified (~ 1.0 mg/ml) by UV-photospectrometry with absorbance at 260 nm. The overall quality and yield of the RNA preparation was determined by denaturing agarose (1 %) gel electrophoresis fractionation. In addition, RNA integrity was confirmed using an Agilent Bioanalyzer (Agilent Technologies, CA, USA). Microarray experiments were performed at the 'McGill University and Génome Québec Innovation Centre', using the Affymetrix (Santa Clara, CA) Rat Genome 230 (version 2.0) GeneChip containing 31,099 probe sets targeting over 28,000 well-characterized rat genes. Synthesis of cDNA and biotin-labelled cRNA, quality control, fragmentation, array hybridization, streptavidin phycoerythrin conjugate staining, washing, and scanning were performed according to standard Affymetrix protocols (GeneChip Analysis Technical Manual, Rev. 5, Affymetrix Inc., 2004). Twelve independent microarray experiments were performed using total hippocampal RNA preparations from twelve different animals (n = 3 animals/treatment). One "biotinylated riboprobe" was loaded per chip. Bioconductor software (Gentleman et al., 2004) processed raw data for background adjustment, quantile normalization and summarization using log scale Robust Multichip Averaging (RMA) (Irizarry et al., 2003). RMA-obtained log2-transformed expression values were imported into a Microsoft Excel® spreadsheet. Statistically significant differences between the experimental and control groups were established using a Student t-test. Genes were considered differentially expressed if they showed 1) average log2(Treated/Control) ratio (ALR) between experimental groups $>|0.585|$ and 2) reported a t-test p-value <0.05.

Hierarchical clustering was performed on log2-transformed values of probe sets showing differential expression in all 4 comparisons between experimental groups. The clustering was done using the Genesis software (IBMT-TUG, Austria) (Sturn et al., 2002) in two dimensions (e.g., both genes and samples) using Euclidian distance as the metric and average linkage.

4.13. *Semi-quantitative reverse transcription PCR (RT-PCR)*

Whole brains were removed (as described in section 4.7) from pup (PND 6) and adult (PND 90) male offspring and the hippocampal tissue was dissected, snap-frozen on dry ice and stored at -80 °C. Total hippocampal RNA was isolated with the Trizol reagent method (Invitrogen, Burlington, Canada) and the precipitated RNA was dissolved in RNase-Free H_2O and quantified (~ 1.0 mg/ml) by UV-photospectrometry with absorbance at 260 nm. The overall quality and yield of the RNA preparation was determined by denaturing agarose (1 %) gel electrophoresis fractionation. In addition, RNA integrity was confirmed using an Agilent Bioanalyzer (Agilent Technologies, CA, USA). cDNA was synthesized in a 20 µl reaction volume containing 2 µg of total RNA, 40 units of Moloney murine leukaemia virus reverse transcriptase (MBI), 5 µM random primer (Roche Molecular Biochemicals), a 1 mM concentration of each of the four deoxynucleotide triphosphates, and 40 units of RNase inhibitor (Roche Molecular Biochemicals). The mRNA was denatured (5 min, 70 °C), the random primers were annealed (10 min, 25 °C) and mRNA was reverse transcribed (1 h, 37°C). The reverse transcriptase was heat-inactivated (10 min, 72 °C) and the products were stored at -20 °C. The rat hippocampal GR exon 1_7 region was subjected to PCR amplification [forward primer (GenBank™ accession number AJ271870): 1941-TCCCAGGCCAG-1949^4867-TTAATATTTGC-4877; reverse primer (GenBank™ accession number NM_012576): 558-TTGAACTCTTGGGGTTCTCTGG-537]. Please note, to select for mRNA transcripts expressed from the exon 1_7 promoter, the forward primer sequence is split into two sections. The first section (1941-TCC CAG GCC AG-1949) of the forward primer is selective for the exon 1_7 region, whereas the second section (4867-TTA ATA TTT GC-4877) of the forward primer sequence is selective for the constitutively expressed exon 2 region. The forward primer design allowed the selection of full-length GR mRNA transcripts containing the exon 1_7 sequence. Because the sequence between the two

sections of the forward primer is unamplified, the PCR product is only 514 bp. To control for equal loading the rat hippocampal β-actin exon region (GenBank™ accession number V01217) was also subjected to PCR amplification (forward primer: 5′-GTTGCTAGCCAGGCTGTGCT-3′; reverse primer: 5′-CGGATGTCCACGTCACACTT-3′). The GR exon 1_7 and β-actin amplification were performed in parallel, using a 25 µl reaction mixture containing 1.5 µl of synthesized cDNA product, 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of *Taq* polymerase (all from MBI) and 0.5 µM of each primer. The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 20-30 cycles of denaturation (30 sec, 95 °C), annealing (30 s, 60 °C) and extension (30 sec, 72 °C), followed by a final extension cycle (5 min, 72 °C) terminating at 4 °C. Samples were removed every 2 cycles between 20 and 30 cycles to determine the linear range of the PCR amplification. Products were separated on an agarose gel (2 %) and post-stained with EtBr to visualize bands corresponding to the GR exon 1_7 (514 bp) or β-actin (470 bp) cDNA fragments. Nucleic acids were transferred by Southern blot (14 h, 22 °C) to positively charged nylon transfer membrane (Hybond-N^+, Amersham). An oligonucleotide (20 bp) specific for the GR exon 1_7 sequence (GenBank™ accession number AJ271870; forward: 5′-TCCCAGGCCAGTTAATATTTGC-3′) was synthesized, as well as an oligonucleotide (21 bp) specific for the β-actin sequence (GenBank™ accession number V01217; forward: 5′-GTTGCTAGCCAGGCTGTGCT-3′). The oligonucleotides were radiolabelled (1 µl T4 polynucleotide kinase (PNK), Promega) with 5 µl [γ-^{32}P]ATP (Amersham) (2 h, 37 °C) and then hybridized to the membranes, which were exposed to phosphorimager screens (Type BAS-III Imaging Plate, Fuji) overnight at 22 °C. The screens were scanned by phosphorimager (Storm840, Molecular Dynamics) running Storm840 software (Molecular Dynamics). Relative optical density (ROD) readings were determined using a computer-assisted densitometry program (ImageQuant, Molecular Dynamics). To control for equal loading between samples, the signal of the GR exon 1_7 region was divided by the signal from the β-actin region amplified from the same sample.

4.14. *Quantitative reverse transcription real-time PCR (QRT-PCR)*

Whole brains were removed (as described in section 4.7) from adult (PND 90) male offspring and the hippocampal tissue was dissected, snap-frozen on dry ice and stored at -80

°C. Total hippocampal RNA was isolated with the Trizol reagent method (Invitrogen, Burlington, Canada) and the precipitated RNA was dissolved in RNase-Free H_2O and quantified (~ 1.0 mg/ml) by UV-photospectrometry with absorbance at 260 nm. The overall quality and yield of the RNA preparation was determined by denaturing agarose (1 %) gel electrophoresis fractionation. In addition, RNA integrity was confirmed using an Agilent Bioanalyzer (Agilent Technologies, CA, USA). Samples were analyzed in triplicate. For each sample, first-strand cDNA synthesis was performed using 5.0 μg RNA preparations according to the manufacture's guidelines (ReactionReady™ First Strand cDNA Synthesis Kit, SuperArray Bioscience Corporation, USA). Pre-designed primers were employed for amplifying ischemia related factor (vof-16, UniGene™ accession number: Rn.38750), alpha thalassemia/mental retardation syndrome X-linked (ATRX, UniGene™ accession number: Rn.107838) and Reelin (Reln, UniGene™ accession number: Rn.98353) RNA transcripts as rendered on the rat 230_2 chip [RT^2 Real-Time™ Gene Expression Assay Kits (version 2.0), SuperArray Bioscience Corporation, USA]. Primers for β-actin (Actb, UniGene™ accession number: Rn.94978) were used for normalization. PCR mixtures (20 μl), containing the first-strand cDNA product, 10 mM Tris-Cl, 50 mM KCl, 2.0 mM MgCl2, 0.2 mM dNTPs, 5X SYBR® Green I Solution, HotStart Taq DNA polymerase and 0.4 μM primer, were loaded into LightCycler capillaries (Roche Molecular Biochemicals). The real-time thermocycler protocol (LightCycler Software 3.5, Roche Molecular Biochemicals) involved an initial HotStart Taq DNA polymerase activation cycle (15 min, 95 °C, with a temperature transition rate set at 20 °C/sec), 35 cycles of denaturation (30 sec, 95 °C, with a temperature transition rate set at 20 °C/sec), annealing (30 sec, 55 °C, with a temperature transition rate set at 20 °C/sec) and elongation (30 sec, 72 °C, with a temperature transition rate set at 2 °C/sec). A single fluorescence reading was acquired at the end of each elongation step. Arithmetic background subtraction was used, and the fluorescence channel was set to F1. To determine the Ct for both the gene of interest (either ATRX, Reelin, or Vof-16) and β-actin, a five-point calibration curve of increasing amounts of RNA (0.5, 1, 2, 4 and 8 μl) as well as a no-template negative control was performed using separate tubes for each reaction (for each dilution and for each gene). The relative amount of both gene transcripts in each sample was determined by plotting the Ct value for each gene on the Y-axis and the log of the amount of RNA used for each reaction on the X-axis. To calculate fold-change

in gene expression, the relative amount of the gene of interest (either ATRX, Reelin, or Vof-16) was divided by the relative amount of β-actin for each sample. The specificity of the amplified PCR products was assessed by performing a melting curve analysis cycle after the PCR amplification (30 sec, 95 °C, with a temperature transition rate set at 20 °C/sec; 30 sec, 65 °C, with a temperature transition rate set at 20 °C/sec; and 95 °C, with a temperature transition rate set at 0.2 °C/sec) that terminated with a cooling step (30 sec, 40 °C, with a temperature transition rate set at 20 °C/sec). The fluorescence of the SYBR® Green I dye bound to double-stranded amplified product declines sharply as the fragment is denatured. The melting temperature of this fragment was visualized by plotting the first negative derivative (dF/dT) of the melting curve on the Y-axis and temperature (°C) on the X-axis. No primer-dimers were detected that interfered with the quantification of the PCR products. The amplified products were also separated on an agarose gel (2 %) and post-stained with EtBr to visualize bands corresponding to vof-16 (190 bp), ATRX (157 bp), Reelin (170 bp) or β-actin (207 bp) cDNA fragments, which were then photographed with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

4.15. *Sodium bisulfite mapping*

Whole brains were removed (as described in section 4.7) from male offspring and the hippocampal tissue was dissected, snap-frozen on dry ice and stored at -80 °C. Sodium bisulfite mapping was performed using the procedure (Frommer et al., 1992) in which sodium bisulfite solution [NaBis (3.6 M)/hydroquinone (1 mM)] was added to the re-suspended DNA and incubated (14 h, 55 °C). DNA was eluted (QIAquick, PCR Purification Kit, QIAGEN) in Tris buffer (10 mM, pH 8.5), and NaOH (3M) was added to a final concentration of 0.3N NaOH and incubated (15 min, 37 °C). NH_4OAc (10 M) was added to a final concentration of 3M NH_4OA, followed by the addition of tRNA (0.1 mg/ml) and EtOH (2 v/v, 95 %) were added and the bisulfited DNA solution was cooled (20 min, -20 °C). The precipitated solution was then centrifuged (4 °C, 13,200 rpm, 30 min) and the pelleted DNA was lyophilized and re-suspended in ddH_2O (50 ng/ ml). The rat exon 1$_7$ glucocorticoid receptor promoter region (GenBank™ accession number: AJ271870) of the sodium bisulfite-treated hippocampal DNA (50 ng/ml) was subjected to PCR amplification using outside primers (Forward: 1646-TTTTTTAGGTTTTTTTAGAGGG-1667; Reverse: 1930-

ATTTCTTTAATTTCTCTTCTCC-1908). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (2 min 30 sec, 59.1 °C) and extension (1 min, 72 °C), followed by a final extension cycle (5 min, 72 °C) terminating at 4 °C. The PCR product (285 bp) was used as a template for sub-sequent PCR reactions utilizing nested primers (Forward: 1738-TTTTTTTGTTAGTGTGATATATTT-1761; Reverse: 1914-TTCTCCCAAACTCCCTCC-1897). The nested PCR product (177 bp) was purified (QIAquick, PCR Purification Kit, QIAGEN) and the DNA was eluted in Tris buffer (10 mM, pH 8.5). The purified product was separated on a 2 % agarose gel and post-stained with EtBr. The band corresponding to DNA fragment (177 bp) was cut from the gel and DNA was extracted (QIAEX, II Agarose Gel Extraction, QIAGEN), and then eluted in Tris buffer (10 mM, pH 8.5). The PCR prod-uct (177 bp) was then sub-cloned (Original TA cloning kit™, Invitrogen Cooperation), transformed into chemically made competent bacteria and grown on x-gal (40 mg/ml)-treated ampicillin (100 mg/ml, Glibco) plates (16 h, 37 °C). Ten different clones per plate were mini prepped, grown (14 h, 37 °C) in 2 ml of LB treated with ampicillin (100 mg/ml), and re-suspended pellet in STET [Sucrose, TritonX-100, EDTA (0.5 M), Tris (1 M, pH 8.0)]. Lysozyme (10 mg/ml, Roche Diagnostics GmbH) dissolved in Tris buffer (25 mM, pH 8.0) was added to each sample (3 sec, 22 °C), which was subsequently boiled (45 sec, 100 °C). Finally, the recovered DNA was re-suspended pellet in 1xTE /RNase A (10 mg/ml). Ten plasmids, containing the ligated exon 1_7 GR promoter DNA fragment, were sequenced per animal (T7 sequencing kit™, USB, Amersham Pharmacia Biotech), starting from procedure C in the manufacturer's protocol. The sequencing reactions were resolved on a denaturing PAGE run in 1x TBE (3 h, 75 W, 22 °C), and visualized by autoradiogra-phy. The method is based on the selective deamination of cytosine to deoxyuracil by treatment with sodium bisulfite. In contrast to cytosine, 5-methylcytosine (5-mC) does not react with sodium bisulfite and can therefore be distinguished. During PCR amplification of the treated DNA sequence, all deoxyuracils are replaced by thymines. Thus, in the final sequence pattern all non-methylated cytosines appear as thymines while 5-mC residues are displayed as cytosines. The full protocol for this procedure, including chemical reagents and solutions, is described in section 12.2.1.

4.16. *Hippocampal cell cultures and transient transfections*

Hippocampal cell cultures from ED 19-20 Long–Evans rat foetuses were prepared (Banker and Cowan, 1977; Bhatnagar and Meaney, 1995; Mitchell et al., 1992) in minimum essential medium (Gibco) containing foetal calf serum (10%), penicillin (0.1%) and streptomycin (0.1%), and supplemented with Hepes (15 mM), potassium chloride (20 mM) and glucose (55 mM, pH 7.4). Hippocampal tissue was mechanically dissociated in Hank's balanced salt solution buffered (pH 7.4) with Hepes (15 mM), washed, digested with trypsin (2.5 mg/ml; Gibco) and digestion stopped with foetal calf serum (10%). The cells were seeded at a density of $3x10^6$ cells (60 mm) or $8x10^6$ (100 mm) cells on poly-D-lysine (Boehringer-Mannheim)-coated Petri dishes. Two days after seeding, uridine (20 mM) and 5-fluorodeox-yuridine (20 mM) were added to the medium to prevent proliferation of glial cells. Cell cultures were maintained at 37 °C in a humid atmosphere with 5% carbon dioxide. Chemically modified phosphorothioate antisense (170-GCGGGGTGCAGGGGCACACT-151) and scrambled antisense (170-TCACACGGGGACGTGGGGCG-151) oligonucleotide (Biognostik GmbH, Göttingen, Germany) were designed and based on the rat NGFI-A genomic region (GenBank™ accession number M18416). The optimum sequence of 20 bases for generating specific oligonucleotides was searched by analyzing the 5′-upstream regions of the open reading frames of the NGFI-A gene and exactly correspond to the sequences reported by (Kukita et al., 1997). The scrambled sequence with the same base ratio as the antisense was confirmed to have no homology to any sequences reported to date in the GenBank™ DNA database. The cultures received a single treatment of either 5H-T (100 nM), 8-bromo cAMP (10 mM) or 5H-T (100 nM) followed by NGFI-A antisense (1 μM) oligonucleotides. Four days later, the cells were harvested.

4.17. *HEK 293 cell cultures and transient transfections*

In preparation of the GR promoter:luciferase plasmid, the rat exon 1$_7$ GR promoter region (GenBank™ accession number: AJ271870) was subjected to PCR amplification (Forward: 1557-AGACGCTGCGGGGGTG-1572; Reverse: 2555-CGACCTGGCCTGGGAG-1940). The thermocycler protocol involved an initial denaturation cycle (30 sec, 95 °C), 35 cycles of denaturation (30 sec, 95 °C), annealing (30 sec, 56 °C) and extension (30 sec, 72 °C),

followed by a final extension cycle (5 min, 72 °C) terminating at 4 °C. The amplified DNA fragment was cloned into a pCR2.1 plasmid (Original TA cloning kit□, Invitrogen Cooperation) and then sub-cloned into a pGL2 plasmid. Accordingly, the pCR2.1 plasmid was digested with *HindIII* then *EcoRI*, and the pGL2 plasmid was digested with *BamH1* then *HindIII*. The released exon 1_7 fragment was then ligated 5′ to 3′ at *Hind III* and *BamHI* sites or 3′ to 5′ at *Xba* and *Hind*III sites within the pGL2 plasmid. *In vitro* mutation of the exon 1_7 GR promoter was performed using the QuikChange® Site-Directed Mutagenesis Kit (Cat#200518, Stratagene®) and following the manufacture's guide. Mutagenesis primers were designed for cytosine to adenine conversion within the 5′ CpG (site 16) dinucleotide (Forward: 5′-CTCGGAGCTGGGAGGGGGGGGGAGG-3′; Reverse: 5′-CCTCCCGCCCCCTCCCAGCTCCGAG-3′) and 3′ CpG (site 17) dinucleotide (Forward: 5′-GCTGGGCGGGGGAGGGAGGGAGCCT-3′; Reverse: 5′-AGGCTCCCTCCCTCCCCCGCCCAGC-3′) in the NGFI-A response element on the exon 1_7 GR promoter (GenBank™ accession number: AJ271870). For *in vitro* methylation, the exon 1_7 GR promoter-pGL2 plasmid (10 μg) was incubated (2 h, 37 °C) with *Sss*I CpG DNA methyltransferase (20 U, New England Biolabs Inc.) in a buffer containing S-adenosylmethionine (SAM, 160 μl). This procedure was repeated twice or until full protection from *Hpa*II digestion was observed. In preparation of the NGFI-A expression plasmid, the NGFI-A coding sequence was sub-cloned into a TOPO-His vector following the manufactures guide (pEF6/V5-His TOPO® TA Expression Kit, Invitrogen). Primers (Forward: 365-GACCATGGACAACTACCCCAAA-384, Tm 66 °C; Reverse: 2553-GCAAATTTCAATTGTCCTAGG-2532, Tm 58 °C) were designed (GenBank™ accession number: M18416). The thermocycler protocol involved an initial denaturation cycle (3 min, 98 °C), 35 cycles of denaturation (30 sec, 98 °C), annealing (30 sec, 55-65 °C) and extension (1 min 30 sec, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. To generate empty control plasmids, the pEF6/V5-His TOPO® construct was digested with *BstXI* to remove the multiple cloning site and then self-ligated. In co-transfection studies, HEK 293 cells were plated at a density of $6x10^4$ in six well dishes and transiently cotransfected with a total amount of 1.5 μg of plasmid DNA (1.0 μg exon 1_7 GR promoter-pGL2 and/or 0.5 μg NGFIA expression plasmid) using the calcium phosphate method as described previously (Rouleau et al., 1992). HEK 293 cells were maintained as

a monolayer in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing foetal calf serum (10 %, Colorado Serum Co.). The cells were harvested 72 h after transfection, lysed and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's protocol. Transfections were repeated a minimum of three times using different cultures of HEK 293 cells.

4.18. *Statistical analysis*

Unless otherwise stated within the text, statistical analyses were performed using analysis of variance (ANOVA) for repeated measures with Tukey's Honestly Significant Difference (HSD) *post-hoc* tests. A computer statistics package (SPSS Inc., USA) was used for data analysis. In general, data are expressed as mean ± SEM within each figure

Chapter 5
Effects of Maternal Care on Hippocampal Expression of BAX and Apoptosis

The experimental procedures and analysed results described in this chapter were published in the *Journal of Neurochemistry* (Weaver et al., 2002). Author contributions: I.C.G.W. and M.J.M. designed research; I.C.G.W. performed research; M.J.M. contributed new reagents/analytic tools; I.C.G.W. analyzed data; and I.C.G.W. and M.J.M. wrote the paper.

The review of literature in Chapter 2 suggests that maternal care influences cognitive development in the rat. Naturally occurring variations in maternal licking/grooming and arched-back nursing (LG-ABN) during the first week of life are associated with differences in spatial-learning/memory that endure with aging (Liu et al., 2000). Spatial-learning and memory in the rat are associated with hippocampal function (Milner et al., 1998; Morris et al., 1982; Whishaw, 1998; Wood et al., 1999), and the first week of life is a period of intense hippocampal synaptogenesis (Gall and Lynch, 1980; Saito et al., 1994). The offspring of mothers that showed increased levels of pup LG and that nursed more frequently in an arched-back posture showed evidence for increased hippocampal synaptogenesis and enhanced spatial learning/memory (Liu et al., 2000). The effect of maternal care on synaptic survival in the neonatal rat hippocampus may be mediated by altered expression of neurotrophic factors; as neonates, the offspring of High LG-ABN mothers show increased hippocampal brain-derived neurotrophic factor (BDNF) expression (Liu et al., 2000). However, there is little understanding of the underlying pathways involved in the preservation of such individual differences in cognitive development, or whether maternal care may influ-

ence neuronal survival during adulthood. Cell division, growth, differentiation and death are finely orchestrated events during normal development. Programmed cell death (i.e. apoptosis) has been suggested to be the orderly demise of cells that have completed their useful function (Ellis et al., 1991; Kerr et al., 1972; Wyllie et al., 1980). Current literature suggests the existence of at least three main apoptosis pathways: the tumour necrosis factor (TNF)-family of death receptors (group I); mitochondria (group II); and endoplasmic reticulum (ER) (group III), with extensive crossover between paths. Among the genes involved in the regulation of these apoptotic pathways, the B-cell lymphoma-2 (BCL-2) proto-oncogene family [consisting of anti-apoptotic members (BCL-2, group II) and pro-apoptotic members (BAX, group I; BAD, group III)] is the best defined (Adams and Cory, 1998; Antonsson and Martinou, 2000; Gross et al., 1999; Oltvai et al., 1993; Yang et al., 1995). Cell death by these proteins is associated with inter-nucleosomal DNA fragmentation (Oppenheim, 1991), often quantified using terminal deoxy-nucleotidyl-transerferase (Tdt)-mediated dUTP biotin nick-end labelling (TUNEL).

5.1. *Experiment A*

In this experiment, we sought to determine whether the activity of genes involved in neuronal survival were influenced by the nature of the early mother-pup interactions, dams were characterized postpartum for frequency of LG-ABN of their offspring during the first week of life. Western blot analysis of BAX, BCL-2 and BAD was performed on the hippocampal tissue from the PND 90 (adult) male offspring of the High and Low LG-ABN dams. TUNEL and DAPI staining was performed on dorsal-hippocampal coronal sections from the adult offspring of the High and Low LG-ABN dams to assess apoptotic activity.

5.1.1. Animals and behaviour

We examined maternal behaviour in Long-Evans dams and separated the PND 90 (adult) male offspring of the High and Low LG-ABN mothers into two cohorts as previously described in section 4.1.

5.1.2. Western blotting analysis

Protein prepared (40 μg/sample), fractionised and transferred as described before in section

4.9. The membranes were blocked (1 h, 22 °C) with Carnation dried milk (5 %) in Tris-buffered saline with Tween (TBS-T) [Tris, NaCl, Tween-20 (0.1 %), pH 7.6], washed briefly in TBS-T and incubated (14 h, 4 °C) with anti-mouse, BAX, BCL-2 or BAD monoclonal primary antibodies in blocking buffer (1: 1000; Santa Cruz Biotech). Membranes were washed with TBS-T (20 min, 22 °C), and then incubated (1 h, 22 °C) with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (1: 2000; Biodesign International). Following four 15-min washes with TBS-T, the bands were visualized by enhanced chemiluminescence (ECL; Amersham) and apposed to autoradiography film (Hyperfilm-MP; Amersham) before being developed. To verify the accuracy of sample loading, selected blots were incubated (30 min, 70 °C) in stripping solution [Tris-HCl (62.5 mM), mercaptoethanol (100 mM), SDS (2 %)] before being blocked (14 h, 4 °C) and reprobed (1 h, 22 °C) with an α-tubulin monoclonal antibody (1: 5000; Biodesign International). A single band was observed at 60 kDa and the intensity of the signal was similar in all lanes. Relative optical density (ROD) readings for the BAX, BCL-2 and BAD bands were determined using a computer-assisted densitometry program (MCID 4.0; Imaging Research) from samples (n = 6 animals/group) run in triplicate on three different blots. For all studies, single blots were derived from samples from one animal.

5.1.3. TUNEL labelling and DAPI counterstaining

Same procedure as described before in section 4.7.

5.1.4. Results

5.1.4.1. Effects of maternal care on hippcampal expression of BAX

Western blot analysis using monoclonal BAX, BCL-2 and BAD antibodies revealed single major bands at 21, 25 and 22 kDa, respectively, in all hippocampal preparations (Figure 15a). The estimated molecular masses are consistent with those previously reported for rat BAX and BCL-2 (Crawley, 1985; Lopez et al., 1999a; Lopez et al., 1999b; Zhu et al., 1999). Quantitative analysis of hippocampal levels of BAX, BCL-2 and BAD immunoreactivity (Figure 15b) revealed a primary effect of treatment [High vs. Low LG-ABN mother; $F(2,5) = 9.5$, $p < 0.006$] and a significant treatment–protein interaction [$F(2,12) = 7.5$, $p < 0.004$]. *Post-hoc* analysis indicated a significant ($p = 0.001$) difference in levels of BAX

immunoreactivity between the offspring of Low and High LG-ABN mothers. In contrast, both BCL-2 and BAD expression did not differ as a function of maternal care.

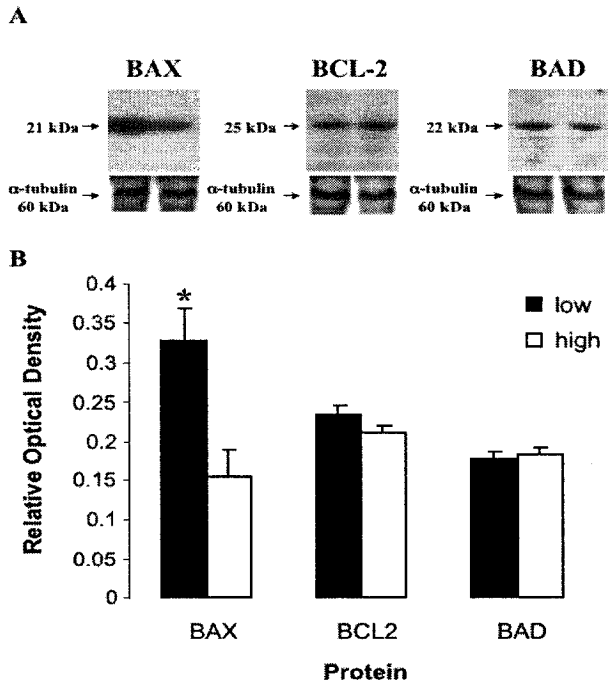

Figure 15. Effects of natural variations in maternal care on BAX, BCL-2 and BAD protein levels in the adult rat hippocampi. (a) A representative western blot illustrating BAX, BCL-2, BAD, and α-tubulin immunoreactivity in the adult offspring of Low (lanes 1, 3 and 5) or High (lanes 2, 4 and 6) LG-ABN mothers. Molecular weight markers corresponded to single major bands at 21, 25 and 22 kDa. (b) Mean ± SEM for BAX, BCL-2 and BAD protein levels expressed as relative optical density (ROD) units from hippocampal samples (n = 6 animals/group) run in triplicate. OD readings were obtained from the same films. *$P < 0.001$.

5.1.4.2. Effects of maternal care on hippcampal cell survival

TUNEL staining was performed on dorsal-hippocampal coronal sections (Figure 16a). Neuronal nuclei were only considered positive for TUNEL if they were round (characteristic of early stage apoptosis) and intensely fluorescent in strong contrast to the surrounding tissue (Figure 16b). Tdt enzyme labelling intensity and background were consistent among slides. Quantitative analysis of hippocampal levels of TUNEL-positive neurones (Figure 16c) are presented as the mean ± SEM number of labelled cells counted from six animals/group (nine sections/animal) and reveal a primary effect of treatment [High vs. Low LG-ABN mother; $F(1,21) = 15.003$, $p < 0.001$] and a main effect of region [dentate gyrus, CA1, CA2 and CA3; $F(3,11) = 31.141$, $p < 0.001$]. However, the interaction of treatment with region was non-significant [$F(1,3) = 2.082$, $p < 0.1$]. *Post-hoc* analysis indicated a

significant increase in the number of TUNEL-positive neurons (Figure 16d) in the dentate gyrus ($p < 0.011$) and CA1 ($p < 0.002$) region of Ammon's horn of the offspring of Low compared with the offspring of High LG-ABN mothers.

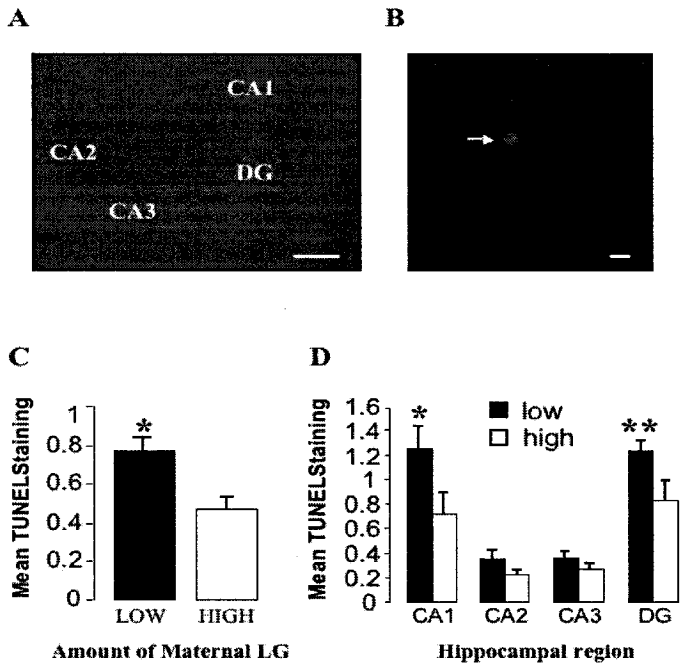

Figure 16. Effects of natural variations in maternal care on the number of TUNEL-positive cells in the adult rat hippocampi. (a) Light-microscope photomicrograph of a dorsal-hippocampal coronal section counterstained with DAPI, illustrating the four different hippocampal fields and level that TUNEL staining was performed. Scale bar, 1.75 mm. (b) Confocal photomicrograph of a representative TUNEL-positive neuron (indicated by the arrow) located within the hillus of the dentate gyrus. Scale bar, 10 μM. (c) Mean ± SEM number of TUNEL-positive neurons counted from hippocampal samples (n = 6 animals/group with nine sections/animal). *$P < 0.001$. (d) Mean ± SEM levels of TUNEL-positive neurons in the dentate gyrus and CA1, CA2 and CA3 hippocampal regions of Ammon's horn. *$P < 0.002$; **$P < 0.011$.

5.2. *Discussion*

Effects of maternal care on brain cell survival

The results of this study demonstrate that anti-apoptotic member BCL-2 and pro-apoptotic members BAX and BAD gene products are detectable in the adult (PND 90) male Long-Evans rat hippocampi. Furthermore, BAX expression is significantly greater in the hippocampi of the offspring of Low compared with High LG-ABN mothers. However, because there is no significant difference in BAD expression between the offspring of High and Low LG-ABN mothers, and because BAX is considerably more highly expressed than

BCL-2 in the offspring of Low LG-ABN mothers, the BAX : BCL-2 ratio may be a more important factor in determining cell fate. TUNEL staining on brain tissue slices allows the simultaneous assessment of cellular morphology and the localization of single cells in situ expressing damaged DNA with either blunt 3′ over-hang or recessed 3′-OH over-hangs. Although only a few nuclei of neurons in each section were labelled, the number of TUNEL-positive cells was significantly greater in the hippocampi from offspring of Low compared with High LG-ABN mothers. Although it remains unclear whether DNA fragmentation occurs as a result of apoptotic and/or necrotic cellular processes (Barth et al., 2000), the neuronal death in this model had apoptotic morphology and showed non-random DNA fragmentation. These findings imply that maternal care affects the hippocampal BAX : BCL-2 ratio and may therefore influence neuronal death in adulthood. Although a wide range of environmental stimuli profoundly affects development in mammals, those provided by the mother are certainly the most critical for early survival and growth (Butler and Schanberg, 1977; Evoniuk et al., 1979; Kuhn et al., 1978; Kuhn et al., 1979; Pauk et al., 1986). In the rat pup, tactile stimulation derived from maternal LG-ABN alters the hippocampal expression of inducible immediate early genes that promote cell cycle progression and the biosynthesis of molecules necessary for normal growth (Heby and Emanuelsson, 1981; Marton, 1987; Slotkin and Bartolome, 1986; Wang et al., 1996). Tactile stimulation also has an immediate impact on endocrine function in the infant rat, including the stimulation of neurotrophic factors and growth hormone (Schanberg et al., 1984), and the inhibition of the highly catabolic GCs (Levine, 1994). Although the current findings do not address the issue of the absolute levels of tactile stimulation required for the long-term regulation of BAX, the data do suggest differences in maternal care can serve as a source of variation in the expression of BAX in the offspring. The potential role of apoptosis during neuronal development includes optimization of synaptic connections, removal of unnecessary neurons and pattern formation, which suggests that neurones are initially overproduced and then compete for target-derived neurotrophic factors (Burek and Oppenheim, 1996; Cowan et al., 1984). We previously reported that the offspring of Low LG-ABN mothers have decreased hippocampal expression of neurotrophic factors relative to offspring of High LG-ABN mothers (Liu et al., 2000). In concert, both neurotrophic factor withdrawal and over-expression of BAX (as reported here in the offspring of Low LG-ABN mothers)

would be expected to promote apoptosis (Oltvai and Korsmeyer, 1994; Oltvai et al., 1993). Indeed, the aging process in the developing rat is related to hippocampal cell death (Kadar et al., 1990). However, in order to examine this hypothesis, a detailed time-course study of BAX and neurotrophic factor expression in the developing rat brain would be invaluable.

Concluding remarks

In summary, I have shown an increased expression of the pro-apoptotic protein BAX and a greater number of TUNEL-positive neurons in the hippocampi from offspring of Low compared with High LG-ABN mothers. These observations lead us to believe that the differences in BAX expression may be involved in differential aging of the hippocampus. Interestingly, we have previously shown that the adult offspring of High LG-ABN mothers perform better in paradigms that test hippocampal-dependent memory and learning, compared with the adult offspring of Low LG-ABN mothers (Liu et al., 2000).

Chapter 6
Epigenetic Programming by Maternal Behaviour

The experimental procedures and analysed results described in this chapter were published in *Nature Neuroscience* (Weaver et al., 2004). Author contributions: I.C.G.W., M.S. and M.J.M. designed research; I.C.G.W. performed research; M.S. and M.J.M. contributed new reagents/analytic tools; I.C.G.W. analyzed data; and I.C.G.W., M.S. and M.J.M. wrote the paper.

The studies in Chapter 5 suggest that maternal care influences neurone survival and hippocampal development in the rat. Through undefined epigenetic processes, maternal effects influence the development of defensive responses to threat in organisms ranging from plants to mammals (Agrawal, 2001; Rossiter, 1999). In the rat, such effects are mediated by variations in maternal behaviour, which serve as the basis for the transmission of individual differences in stress responses from mother to offspring (Fleming et al., 1999; Levine, 1994; Meaney, 2001). Mother-pup contact in the rat primarily occurs within the context of a nest bout, which begins when the mother approaches the litter, licks and grooms her pups, and nurses while occasionally licking and grooming the pups (Stern, 1997). There are stable individual differences in two forms of maternal behaviour over the first week of lactation, termed licking/grooming and arched-back nursing (LG-ABN) (Caldji et al., 1998; Francis et al., 1999; Liu et al., 1997; Myers et al., 1989a; Stern, 1997). Such naturally occurring variations in maternal behaviour are associated with the development of individual differences in behavioural and HPA responses to stress in the offspring. As

adults, the offspring of High LG-ABN mothers are less fearful and show more modest HPA responses to stress than the offspring of Low LG-ABN mothers (Caldji et al., 1998; Francis et al., 1999; Liu et al., 1997; Myers et al., 1989a; Stern, 1997). Cross-fostering studies show that the biological offspring of Low LG-ABN mothers reared by High LG-ABN dams resemble the normal offspring of High LG-ABN mothers [and vice versa (Francis et al., 1999)]. These findings suggest that variations in maternal behaviour serve as a mechanism for the non-germ-line transmission of individual differences in stress reactivity across generations (Fleming et al., 1999; Francis et al., 1999; Meaney, 2001). The critical question concerns the mechanisms whereby these maternal effects, or other forms of environmental programming, are sustained over the lifespan of the animal.

Maternal behaviour in the rat permanently alters the development of HPA responses to stress through tissue-specific effects on gene expression. The magnitude of the HPA response to acute stress is a function of hypothalamic corticotrophin-releasing hormone (CRH) release, which activates the pituitary-adrenal system. There are also modulatory influences, such as GC negative feedback that inhibits CRH synthesis and release, thus dampening HPA responses to stress (De Kloet et al., 1998). The adult offspring of High compared with Low LG-ABN mothers show increased hippocampal GR expression and enhanced GC feedback sensitivity (Francis et al., 1999; Liu et al., 1997). Predictably, adult offspring of High LG-ABN mothers show decreased hypothalamic CRH expression and more modest HPA responses to stress (Liu et al., 1997). Eliminating the difference in hippocampal GR levels abolishes the effects of early experience on HPA responses to stress in adulthood (Meaney et al., 1989), suggesting that the difference in hippocampal GR expression serves as a mechanism for the effect of early experience on the development of individual differences in HPA responses to stress (Meaney, 2001).

In vivo and *in vitro* studies suggest that maternal LG-ABN increase GR gene expression in the offspring through increased serotonin (5-HT) activity at 5-HT_7 receptors, and the subsequent activation of cAMP and cAMP-dependent protein kinase activity (Laplante et al., 2002; Meaney et al., 2000; Weaver et al., 2001). Both the *in vitro* effect of 5-HT and the *in vivo* effect of maternal behaviour on GR gene expression are accompanied by an increased hippocampal expression of the transcription factor nerve growth factor-

inducible protein A (NGFI-A). The non-coding exon 1 region of the hippocampal GR includes a promoter region, exon 1_7, containing a binding site for NGFI-A (McCormick et al., 2000). Splice variants of the GR mRNA containing the exon 1_7 sequence are found predominantly in the brain, and the expression of GR mRNAs containing the exon 1_7 sequence is increased in the offspring of High LG-ABN mothers or following manipulations that increase maternal licking and grooming (McCormick et al., 2000; Weaver, 2001), suggesting that the use of this promoter is enhanced as a function of maternal care. Although these findings might explain the increased GR expression in the neonate, we are left with the question of how the effect of maternal care might persist into adulthood. Gene expression is controlled by the epigenome, which is comprised of chromatin structure (Kadonaga, 1998) and DNA methylation (Razin, 1998).

6.1. *Experiment A*

In this experiment, we sought to determine whether maternal care alters DNA methylation of the GR exon 1_7 promoter, and whether these changes are stably maintained into adulthood and associated with differences in GR expression and HPA responses to stress.

6.1.1. Animals and behaviour

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Catherine's, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. For further methodological details, see sections 4.1 and 4.2. For the sake of the developmental study, cohorts of female offspring of High or Low LG-ABN mothers were mated. Pups were taken at ED 20 or on PND 1, 6, 21 or 90 (with the day of birth = PND 1). The behavioural status of the mothers was confirmed across the groups using mothers of pups taken at PND 6-90. Analysis revealed that the frequency of licking/grooming and arched-back nursing in mothers derived from High LG-ABN dams was significantly higher than that of mothers derived from Low LG-ABN dams.

6.1.2. Sodium bisulfite mapping

Same procedure as described before in section 4.15.

6.1.3. Chromatin immunoprecipitation (ChIP) assay

Same procedure as described before in section 4.10.

6.1.4. Results

6.1.4.1. Maternal care and methylation of exon 1_7 promoter

DNA methylation is a stable, epigenomic mark at CpG dinucleotides often associated with stable variations in gene transcription (Keshet et al., 1985; Razin, 1998; Razin and Cedar, 1977). Two kinds of changes in DNA methylation are known to affect gene expression: regional, non-site specific DNA methylation around a promoter (Keshet et al., 1985) and site-specific methylation. Hypomethylation of CpG dinucleotides of regulatory regions of genes is associated with active chromatin structure and transcriptional activity (Razin, 1998; Razin and Cedar, 1977). Thus, the methylation pattern is a stable signature of the epigenomic status of a regulatory sequence. We focused on the methylation state of the exon 1_7 GR promoter, which is activated in the hippocampus in offspring of High LG-ABN mothers.

To determine whether DNA methylation of specific target sites on the GR promoter change in response to maternal care, we mapped differences in the methylation status of individual cytosines within the CpG dinucleotides of the exon 1_7 promoter from hippocampal tissue from the adult offspring of High and Low LG-ABN mothers. We used sodium bisulfite mapping (Clark et al., 1994; Frommer et al., 1992), with a particular interest in the region around the NGFI-A consensus sequence (Figure 17a). The results showed significant differences in the methylation of specific sites of the exon 1_7 GR promoter sequence (Figure 17b,c). A two-way ANOVA revealed a highly significant effect of Group [$F(1,136) = 55.9$, $P < 0.0001$] and Region [$F(16,136) = 27.7$, $P < 0.0001$], as well as a significant Group x Region interaction effect [$F(16,136) = 27.7$, $P < 0.0001$]. Importantly, the cytosine residue within the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence (Figure 17c) is always methylated in the offspring of Low LG-ABN mothers, and rarely methylated in the offspring of High LG-ABN dams. In contrast, the 3′ CpG dinucleotide (site 17) remains methylated, regardless of differences in maternal care. Dissected hippocampi inevitably contain glial cells as well as neurons. Considering the pronounced effect of maternal care on the methylation status of the 5′ CpG dinucleotide of the NGFI-A

response element (> 90 %), the effect of maternal care must include neuronal as well as glial cells; both populations express GR (Cintra et al., 1994a; Cintra et al., 1994b) and NGFI-A (Brinton et al., 1998) genes.

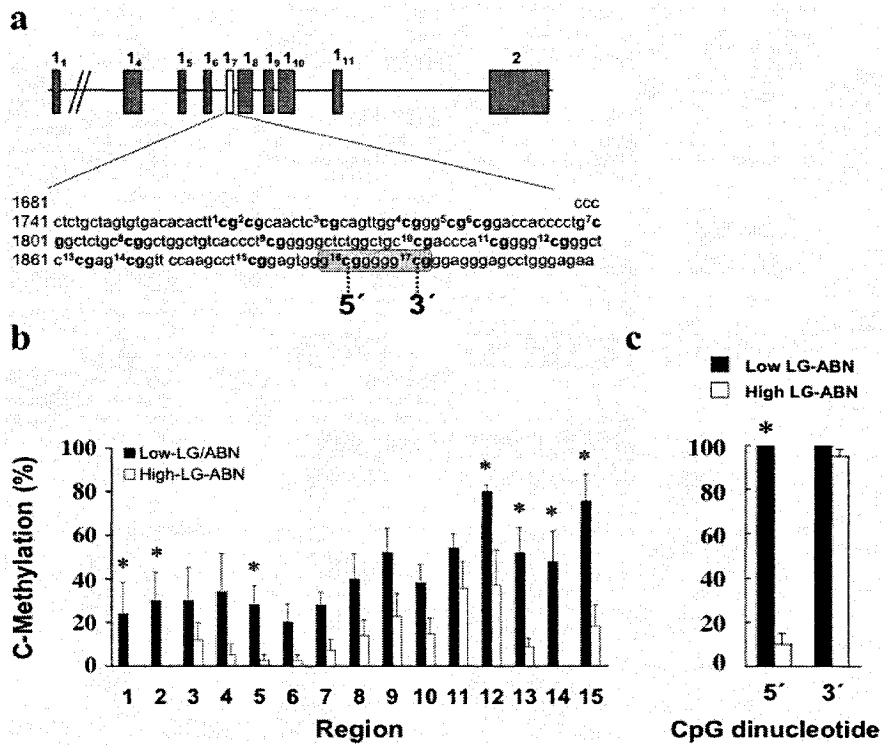

Figure 17. Maternal care alters cytosine methylation of GR promoter. (a) Sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (bold) and the NGFI-A binding region 16 (encircled). (b,c) Methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from adult High and Low LG-ABN offspring (6-10 clones sequenced/animal; n = 4 animals/group; *$P < 0.01$). (b) Percentage of cytosine residues that were methylated (mean ± SEM) for the first 15 CpG dinucleotides (*$P < 0.05$). (c) Percentage of methylated cytosines (mean ± SEM) for the 5′ (site 16) and 3′ (site 17) CpG dinucleotides within the NGFI-A binding sequence (*$P < 0.0001$).

6.1.4.2. Cross-fostering reveals epigenetic marking by maternal behaviour

Our findings suggest that specific sites within the exon 1_7 GR promoter are differentially methylated as a function of maternal behaviour, but these findings are merely correlational. To directly examine the relation between maternal behaviour and DNA methylation within the exon 1_7 promoter, we performed an adoption study in which the biological offspring of High or Low LG-ABN mothers were cross-fostered to either High or Low LG-ABN dams within twelve hours of birth (Francis et al., 1999). Cross-fostering produced a pattern of exon 1_7 promoter methylation that was associated with the rearing mother [$F(3,544) = 4.8$,

$P < 0.05$; Figure 18] and thus reversed the difference in methylation at specific cytosines, notably at the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence (Figure 18, left panel). Thus, in the Low LG-ABN offspring that were fostered to High LG-ABN dams, methylation of this 5′ site within the exon 1_7 promoter was indistinguishable from that of the biological offspring of High LG-ABN mothers. Likewise, the methylation of the same 5′ CpG dinucleotide in the biological offspring of High LG-ABN mothers reared by Low LG-ABN dams was comparable to that of Low LG-ABN offspring. There was no effect of cross-fostering at the cytosine within the 3′ CpG dinucleotide (site 17; Figure 18).

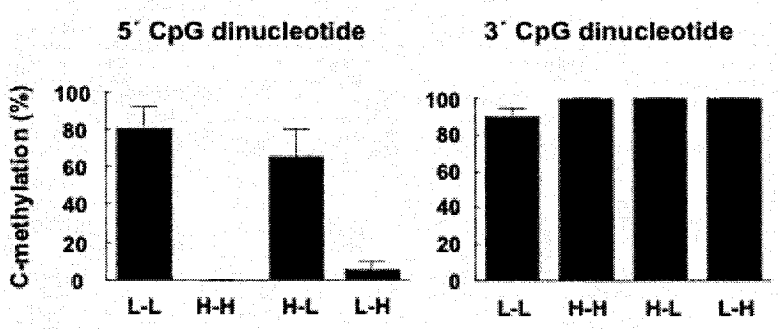

Figure 18. The effect of cross-fostering the offspring of High and Low LG-ABN mothers on cytosine methylation of the 5′ and 3′ CpG dinucleotides within the NGFI-A binding sequence of the exon 1_7 GR promoter gene in adult hippocampi (n = 5 animals/group). L-L: animals born to and reared by Low LG-ABN mothers; H-H: animals born to and reared by High LG-ABN mothers; H-L: animals born to High LG-ABN mothers and reared by Low LG-ABN mothers; L-H: animals born to Low LG-ABN mothers and reared by High LG-ABN mothers.

These findings suggest that variations in maternal care directly alter the methylation status of the exon 1_7 promoter of the GR gene. Thus, we have demonstrated that a DNA methylation pattern can be established through a behavioural mode of programming without germ line transmission. In parental imprinting, a well-established paradigm of inheritance of an epigenomic mark, the paternally and maternally inherited alleles are differentially methylated. These methylation patterns are defined during maturation of spermatocytes and oocytes, and are transmitted to the offspring through the germ line (Sapienza, 1990).

6.1.4.3. Timing of the maternal effect on DNA methylation

The maternal care of High and Low LG-ABN mothers differs only during the first week of life (Caldji et al., 1998; Liu et al., 1997). Thus, we wondered whether this period corre-

sponds to the timing for the appearance of the difference in DNA methylation in the offspring. We used sodium bisulfite mapping to examine the methylation status of the cytosines within the exon 1_7 GR promoter during development (Figure 19). Statistical analysis of the data for the 5′ CpG (site 16) revealed a highly significant effect of Group [$F(1,544) = 66.7$, $P < 0.0001$) and Age [$F(1,544) = 21.1$, $P < 0.0001$] as well as a significant interaction effect [$F(1,544) = 13.7$, $P < 0.0001$]. Tukey's *post-hoc* analysis revealed that the Group effect on methylation status of the 5′ CpG (site 16) was significant at P6, P21 and P90 ($P < 0.001$), but not at E20 or P1. Just before birth (ED 20; E20), the entire region was unmethylated in both groups. Strikingly, one day after birth (PND 1; P1) the exon 1_7 GR promoter was *de novo* methylated in both groups. The 5′ and 3′ CpG sites of the exon 1_7 GR NGFI-A response element in the offspring of both High and Low LG-ABN mothers, which exhibit differential methylation later in life, were *de novo* methylated to the same extent. These data show that both the basal state of methylation and the first wave of *de novo* methylation after birth occur similarly in both groups. Whereas it is generally accepted that DNA methylation patterns are formed prenatally and that *de novo* methylation occurs early in development, there is at least one documented example of postnatal *de novo* methylation of the *Hoxa5* and *Hoxb5* genes (Hershko et al., 2003). Because similar analyses are not documented for other genes, it remains unknown whether changes in methylation are common around birth or whether they are unique to this GR promoter.

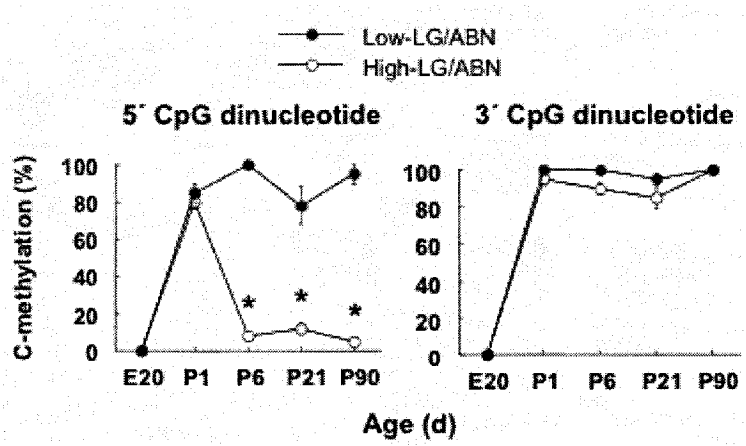

Figure 19. Percentage of cytosine methylation (mean ± SEM) of the 5′ and 3′ CpG dinucleotides within the NGFI-A binding region of the exon 1_7 GR promoter gene in the offspring of High or Low LG-ABN mothers (n = 5 animals/group; P < 0.001) as a function of age. There were no differences at any postnatal age in level of cytosine methylation of the 3′ CpG (site 17).

The differences in the methylation status of the exon 1_7 GR promoter between the two groups developed between P1 and P6, the period when differences in the maternal behaviour of High and Low LG-ABN dams are apparent (Caldji et al., 1998; Meaney, 2001). By P6, the NGFI-A response element 5′ CpG dinucleotide (site 16) was effectively 'demethylated' in the High, but not in the Low LG-ABN group. The group difference in CpG dinucleotide methylation remains consistent through to adulthood (P90; Figure 19). These findings, together with those of the cross-fostering study, suggest that the group difference in DNA methylation occurs as a function of a maternal behaviour over the first week of life. The results of earlier studies indicate that the first week of postnatal life is a 'critical period' for the effects of early experience on hippocampal GR expression (Meaney et al., 1996). For a full analysis of the time-line study, including methylation patterns of all 17 CpG dinucleotides within the exon 1_7 GR promoter, see section 12.1.

6.1.4.4. Maternal effects on chromatin structure and NGFI-A binding

The next question concerns the functional importance of such differences in methylation. DNA methylation is associated with changes in chromatin activity states (Razin, 1998). Chromatin gates the accessibility of promoters to transcription factors (Kadonaga, 1998). Histone acetylation at the lysine-9 (K9) residue of H3 and H4 histones is a well-established marker of active chromatin (Kadonaga, 1998; Roth et al., 2001). Acetylation of the histone tails neutralizes the positively charged histones, which disrupts histone binding to negatively charged DNA and thus promotes transcription factor binding. We tested the hypothesis that the maternal effect on DNA methylation results in (i) increased histone acetylation at the K9 residue of the H3 histone(s) associated with the exon 1_7 GR promoter and (ii) increased interaction between NGFI-A and the promoter sequence. We performed a chromatin immunoprecipitation (ChIP) analysis of histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter in the native chromatin environment *in vivo*. Intact hippocampi from adult offspring of High and Low LG-ABN mothers were cross-linked *in vivo* by para-formaldehyde perfusion. We then selectively immunoprecipitated protein-DNA complexes with either an acetylated H3-K9 histone primary antibody or an NGFI-A primary antibody. The protein-DNA complexes were uncrosslinked, and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence. There were significant Group effects for the association of

both histone H3-K9 acetylation [*t(1,16) = 2.1, *P < 0.001*] and NGFI-A [*t(1,16) = 3.1, **P < 0.0001*] with the exon 1_7 GR promoter sequence. These results indicated that significantly greater histone H3-K9 acetylation association and threefold greater binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the adult offspring of High compared with Low LG-ABN mothers (Figure 20). Thus, maternal programming of the exon 1_7 GR promoter involves DNA methylation, histone H3-K9 acetylation and alterations in NGFI-A binding.

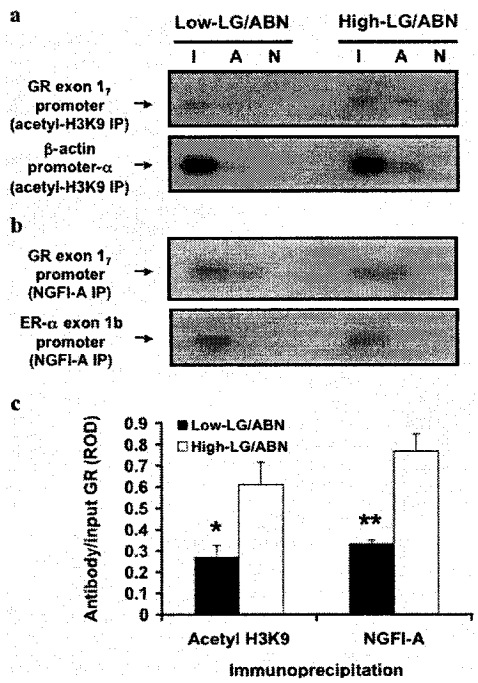

Figure 20. Chromatin immunoprecipitation analysis of the association between histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR sequence in hippocampal tissue from adult offspring of High and Low LG-ABN mothers (n = 4 animals/group). (a,b) Lanes were loaded with non-immunoprecipitated input (I), acetylated histone H3-K9 (top) or NGFI-A (middle) primary antibody immunoprecipitated (A), or non-immune IgG antibody immuno-precipitated (N) hippocampal extracts). (a) Representative Southern blot of the amplified exon 1_7 region from acetyl-histone H3-K9 immunoprecipitated hippocampal tissue (194 bp band) and β-actin (171 bp band) control. (b) Representative Southern blot of the amplified exon 1_7 region of the GR from NGFI-A immunoprecipitated hippocampal tissue (194 bp band). DNA loading was controlled using primers specific for the ubiquitously expressed β-actin promoter-α region. Exon 1b oestrogen receptor-α promoter region, which does not contain NGFI-A recognition elements (493 bp), amplified from the same NGFI-A immunoprecipitated hippocampal tissue was run as a control for specificity and showed no signal. (c) Relative optical density (ROD; mean ± SEM) of exon 1_7 sequence amplified from acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue of adult High and Low LG-ABN offspring (n = 4 animals/group; **P < 0.001*; ***P < 0.0001*).

These findings suggest that maternal care influences hippocampal GR expression,

and thus HPA function in the offspring, through epigenetic alterations that regulate NGFI-A binding to the exon 1_7 promoter. A critical question is whether the impact of early experience is reversible and whether epigenetic programming is modifiable in adult, post-mitotic tissues. The generally accepted model is that the DNA methylation pattern is an irreversible reaction in adult post-mitotic cells. However, recent data from *in vitro* experiments suggests that in certain instances it is possible to induce replication-independent demethylation of ectopically methylated genes by increasing histone acetylation using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (Cervoni and Szyf, 2001; Roth et al., 2001). Cytosine methylation attracts methylated DNA binding proteins and HDACs that prevent histone acetylation and thus transcription factor binding (Cervoni and Szyf, 2001; Roth et al., 2001). Activation of chromatin through HDAC inhibition might trigger DNA demethylation by increasing the accessibility of DNA to demethylase activity (Cervoni and Szyf, 2001; Roth et al., 2001).

6.2. *Experiment B*

In this experiment, we sought to determine whether the hypothesis that inhibition of HDACs with TSA would result in increased K9 acetylation of H3-histones associated with the exon 1_7 GR promoter, DNA demethylation, NGFI-A binding and reversal of maternal programming of stress responses in the adult offspring of Low LG-ABN mothers.

6.2.1. Animals and behaviour

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Catherine's, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. For further methodological details, see section 4.1.

6.2.2. Intracerebroventricular infusions

Same procedure as described before in section 4.3. In this study, a total volume of 2 µl of TSA (100 ng/ml in DMSO) or DMSO vehicle alone was injected (using a Hamilton 10 µl micro-syringe) through the infusion cannula over a 1-min period.

6.2.3. Sodium bisulfite mapping

Same procedure as described before in section 6.1.2.

6.2.4. Chromatin immunoprecipitation (ChIP) assay

Same procedure as described before in section 6.1.3.

6.2.5. Western blotting analysis

Same procedure as described before in section 4.9.

6.2.6. HPA response to restraint stress

Same procedure as described before in section 4.6.

6.2.7. Results

6.2.7.1. Reversal of maternally mediated epigenetic marking

We first used ChIP analysis to determine whether histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter is altered in the offspring of High and Low LG-ABN mothers through intracerebroventricular (ICV) infusion of the adult offspring with TSA (100 ng/ml) or vehicle. Statistical analysis revealed a significant Group x Treatment interaction effect for both the histone H3-K9 acetylation [$F(1,16) = 4.93$, $P < 0.05$] and NGFI-A [$F(1,16) = 8.97$, $P = 0.01$]. *Post-hoc* analysis showed that for both assays, vehicle -treated offspring of Low LG-ABN mothers showed significantly ($^*P < 0.01$) less association than any other group. These results indicate greater histone H3-K9 acetylation association and more binding (>3 fold) of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the adult offspring of TSA-treated Low LG-ABN mothers compared with the vehicle-treated offspring of Low LG-ABN mothers (Figure 21); there were no significant differences between TSA treated offspring of Low LG-ABN mothers and either TSA- or vehicle- treated offspring of High LG-ABN dams. As expected, TSA treatment did not change histone H3-K9 acetylation or NGFI-A binding in the adult offspring of High LG-ABN mothers, because the GR exon 1_7 promoter region in the offspring of High LG-ABN mothers is normally associated with acetylated histones and highly bound with NGFI-A.

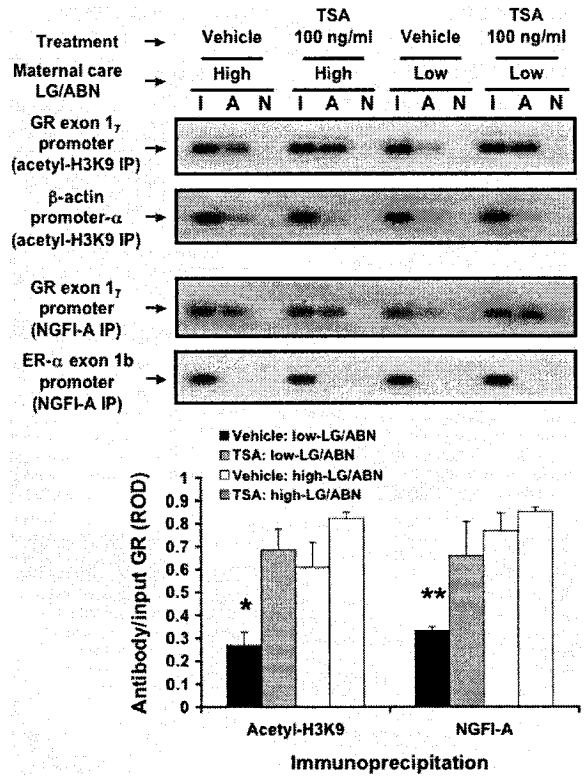

Figure 21. HDAC inhibitor (TSA) eliminates maternal effect on histone acetylation and NGFI-A binding. (a) Chromatin immunoprecipitation analysis of the association between histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR promoter sequence in hippocampal tissue from vehicle- and TSA-treated (100 ng/ml) adult offspring of High and Low LG-ABN mothers (n = 4 animals/group; lane labels as described in Figure 20). (b) Relative optical density (ROD; mean ± SEM) of exon 1_7 sequence amplified from acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue (*$P < 0.05$; **$P < 0.01$).

To determine whether TSA treatment reverses the maternal effect on methylation within specific CpG dinucleotides on the exon 1_7 GR promoter, we mapped the differences in methylation using the sodium bisulfite technique, focusing on the NGFI-A consensus sequence within the exon 1_7 region (Figure 17a). Statistical analysis of the data across all 17 sites revealed a significant effect of Group [$F(1,272) = 93.2$, $P < 0.0001$], Treatment [$F(1,272) = 52.8$, $P < 0.0001$] and Region [$F(16,272) = 30.4$, $P < 0.0001$], as well as a significant Group x Treatment x Region interaction [$F(16,272) = 2.1$, $P = 0.01$], Group x Treatment interaction [$F(1,272) = 19.9$, $P < 0.0001$], Group x Region interaction [$F(16,272) = 4.1$, $P < 0.0001$] and Treatment x Region interaction [$F(16,272) = 2.8$, $P < 0.0001$]. The results again revealed significant differences in the methylation of a number of regions of the exon 1_7 GR promoter sequence (Figure 22) with significant differences within the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides of the NGFI-A consensus

sequence (Figure 22b). Statistical analysis of the data from these two sites revealed a highly significant effect of Group [$F(1,32) = 43.8$, $P < 0.0001$], Treatment [$F(1,32) = 65.3$, $P < 0.0001$) and Region [$F(1,32) = 113.3$, $P < 0.0001$], as well as a significant Group x Treatment interaction [$F(1,32) = 16.0$, $P < 0.0001$], Group x Region interaction [$F(1,32) = 37.8$, $P < 0.0001$] and Treatment x Region interaction [$F(1,32) = 4.5$, $P = 0.04$]. *Post-hoc* analysis revealed that TSA treatment significantly decreased the degree of cytosine methylation within the 5′ (site 16) CpG dinucleotide of the NGFI-A binding region of the exon 1_7 GR promoter in the offspring of Low LG-ABN mothers in comparison to vehicle-treated Low LG-ABN mothers (*$P < 0.001$). TSA treatment produced 'demethylation' of the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides in the offspring of Low LG-ABN mothers, and hypomethylation of the 3′ CpG (site 17) dinucleotide in the offspring of High LG-ABN mothers (Figure 22b). These findings suggest that TSA treatment can reverse the hypermethylated status of the exon 1_7 GR promoter in the offspring of Low LG-ABN mothers. TSA treatment resulted in a more extensive change in DNA methylation than maternal care *per se*, since the 3′ CpG (site 17) dinucleotide, which is unaffected by maternal behaviour, is partially 'demethylated' in response to TSA treatment in both cohorts (Figure 22b). In addition, as in the original study (Figure 17b), maternal care altered the methylation status of other CpG dinucleotides in the exon 1_7 sequence; in the case of sites 1, 2, 5, 12, 14 and 15, these effects were similarly reversed with central TSA infusion. The significance of these sites for transcription factor binding is currently unknown and thus a focus of ongoing studies. Thus, stable DNA methylation marking by maternal behaviour is reversible in the adult offspring hippocampus by pharmacological modulation of chromatin structure. While TSA altered the methylation of the both the 5′ and 3′ CpG sites within the NGFI-A response element, the former appears to be critical for the effect on NGFI-A binding to the exon 1_7 promoter.

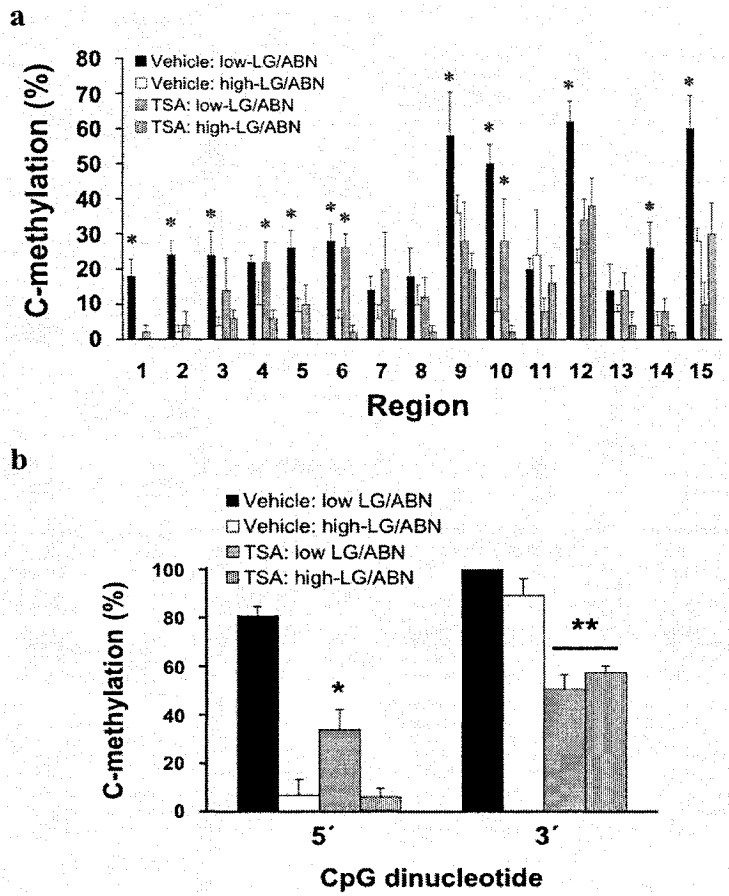

Figure 22. TSA effects on cytosine methylation. (a,b) Methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter in hippocampi of vehicle- and TSA-treated (100 ng/ml) adult offspring of High and Low LG-ABN mothers (n = 5 animals/group). (a) Percentage of cytosine residues that were methylated (mean ± SEM) for the first 15 CpG dinucleotides (*$P < 0.05$). (b) Percentage of methylated cytosines for the 5′ (site 16) and 3′ (site 17) CpG dinucleotides within the NGFI-A binding region (*$P < 0.001$; **$P < 0.003$).

6.2.7.2. Reversal of maternal effect on GR expression

GR gene expression in the hippocampus is increased in the adult offspring of High compared with Low LG-ABN mothers (Francis et al., 1999). We suggest that such differences are mediated by the differential methylation of the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence in the exon 1_7 GR promoter and the subsequent alteration of histone acetylation and NGFI-A binding to the exon 1_7 sequence. If the differential epigenetic marking regulates the expression of the exon 1_7 GR promoter in High versus Low LG-ABN offspring, then reversal of the epigenetic marking should be accompanied by an increase in hippocampal GR expression. This hypothesis is supported by the results (Figure 23) showing that hippocampal GR expression was significantly increased in TSA-

treated offspring of Low LG-ABN mothers to levels that were comparable to those of either the vehicle- or TSA-treated offspring of High LG-ABN mothers. ANOVA revealed highly significant main effects of Group [$F(1,16) = 7.4$, $P = 0.01$] and Dose [$F(1,16) = 24.8$, $P < 0.0001$], as well as a significant Group x Dose interaction effect [$F(1,16) = 3.1$, $P = 0.048$]. *Post-hoc* analysis indicated that 100 ng/ml TSA treatment significantly increases hippocampal GR expression in the offspring of Low LG-ABN mothers (vehicle-treated Low LG-ABN vs. 100 ng/ml TSA treated Low LG-ABN, $*P < 0.001$), such that there is no longer a significant difference in hippocampal GR expression between the offspring of Low or High LG-ABN mothers (100 ng/ml TSA treated Low LG-ABN vs. 100 ng/ml TSA treated High LG-ABN, $P > 0.90$). Although TSA treatment significantly induced GR expression in Low LG-ABN adult offspring, global abundance of protein in the hippocampus was not apparently increased, as indicated by the equal α-tubulin immunoreactivity (Figure 23). In comparing the vehicle-treated groups, note that the effect of maternal care on GR gene expression (Francis et al., 1999; Liu et al., 1997) is subtler than the more pronounced effect on the methylation status of the 5′ CpG dinucleotide (site 16) within the exon 1_7 promoter (Figure 22b). However, in previous studies (McCormick et al., 2000) we found evidence for multiple promoters regulating hippocampal GR expression suggesting that exon 1_7 is but one of the regulatory sequences determining GR expression within the hippocampus.

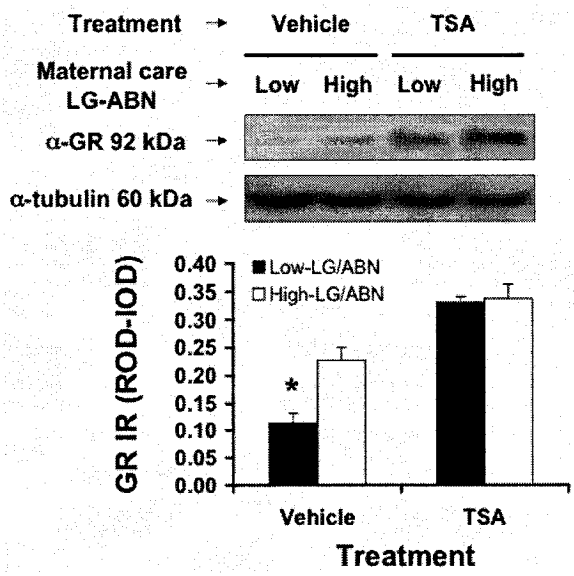

Figure 23. TSA eliminates the maternal effect on hippocampal GR expression. The top panel shows a representative western blot showing absolute levels of electrophoresed hippocampal GR

immunoreactivity (IR) from vehicle- and TSA (100 ng/ml)-treated adult offspring of High or Low LG-ABN mothers. Molecular weight markers (SeeBlue, Santa Cruz Biotech) correspond to a single major band at 92 kDa. The middle panel shows the membrane reprobed for α-tubulin IR, illustrating absolute levels of electrophoresed hippocampal protein bound to the transfer membrane. Molecular weight markers correspond to a single major band at < 60 kDa and the intensity of the signal was similar in all lanes. The lower panel shows quantitative densitometric analysis (relative optical density, ROD) of GR IR levels from samples (n = 5 animals/group; *$P < 0.001$).

6.2.7.3. Reversal of maternal effect on HPA responses to stress

As adults, the offspring of High LG-ABN mothers show more modest HPA responses to stress than the offspring of Low LG-ABN mothers (Liu et al., 1997). The effect of maternal care on HPA responses to stress seems to be, in part, associated with differences in hippocampal GR levels and GC negative feedback sensitivity (Liu et al., 1997). Given that TSA treatment reversed the group difference in hippocampal GR expression, we examined the adrenocortical responses to stress in a separate cohort of vehicle- and TSA-treated animals. Central infusion of TSA eliminated the maternal effect on HPA responses to acute stress (Figure 24). Statistical analysis of the plasma corticosterone data revealed significant effects of Group [$F(1,34) = 4.3$, $P = 0.048$], Treatment [$F(1,34) = 4.3$, $P = 0.046$] and Time [$F(1,34) = 27.3$, $P < 0.0001$], as well as a significant Group x Treatment interaction effect [$F(1,34) = 7.7$, $P = 0.009$]. *Post-hoc* analysis revealed that TSA treatment significantly decreased basal plasma corticosterone in the offspring of Low LG-ABN mothers to levels comparable to those of High LG-ABN animals. Thus, plasma corticosterone responses to restraint stress in the vehicle-treated adult offspring of Low LG-ABN mothers were significantly ($P < 0.01$) greater than those of TSA- and vehicle-treated adult offspring of High LG-ABN mothers or TSA-treated offspring of Low LG-ABN mothers. The HPA response to stress in the offspring of High LG-ABN mothers was unaffected by TSA treatment.

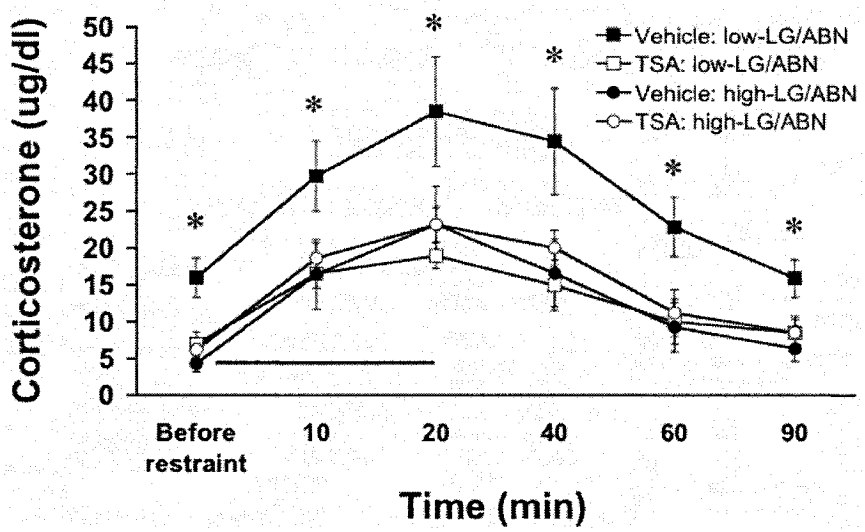

Figure 24. TSA eliminates the maternal effect on HPA responses to stress. Plasma corticosterone responses (Liu et al., 1997) (mean ± SEM) to a 20-min period of restraint stress (solid bar) in vehicle- and TSA (100 ng/ml)-treated adult offspring of High or Low LG-ABN mothers (n = 10 animals/group; *$P < 0.01$).

6.3. *Discussion*

Effects of maternal care on epigenetic progamming of responses to stress

Further studies are required to determine how maternal behaviour alters the epigenetic status of the exon 1_7 GR promoter. In addition, the exact causal relationship between DNA methylation and altered histone acetylation and NGFI-A binding remains to be defined. Nevertheless, our findings provide the first evidence that maternal behaviour produces stable alterations of DNA methylation and chromatin structure, providing a mechanism for the long-term effects of maternal care on gene expression in the offspring. These studies offer an opportunity to clearly define the nature of gene-environment interactions during development and how such effects result in the sustained environmental programming of gene expression and function over the lifespan. It is important to note that maternal effects on the expression of defensive responses, such as increased HPA activity, are a common theme in biology (Agrawal, 2001; Rossiter, 1999) such that the magnitude of the maternal influence on the development of HPA and behavioural responses to stress in the rat should not be surprising. Maternal effects on defensive responses to threat are apparent in plants, insects and reptiles. Such effects commonly follow from the exposure of the mother to the same or similar forms of threat and may represent examples whereby the experience of the

mother is translated through an epigenetic mechanism of inheritance into phenotypic variation in the offspring. Thus, maternal effects could result in the transmission of adaptive responses across generations (Agrawal, 2001; Meaney, 2001; Rossiter, 1999). Indeed, among mammals, natural selection may have shaped offspring to respond to subtle variations in parental behaviour as a forecast of the environmental conditions they will ultimately face once they become independent of the parent (Meaney, 2001). Epigenetic modifications of targeted regulatory sequences in response to even reasonably subtle variations in environmental conditions might then serve as a major source of epigenetic variation in gene expression and function, and ultimately as a process mediating such maternal effects. We propose that effects on chromatin structure such as those described here serve as an intermediate process that imprints dynamic environmental experiences on the fixed genome, resulting in stable alterations in phenotype.

Concluding remarks

In summary, central infusion of the HDAC inhibitor TSA enhanced histone H3-K9 acetylation of the exon 1_7 GR promoter in the offspring of the Low LG-ABN mothers, increased NGFI-A binding to its cognate sequence, induced hypomethylation of CpG dinucleotide sequences in the promoter and eliminated the maternal effect on hippocampal GR expression and the HPA response to stress. These findings are consistent with idea that the maternal effect on GR expression and HPA responses to stress is mediated by alterations in chromatin structure. I propose that the reduced binding of NGFI-A to its response element on the hypoacetylated and hypermethylated exon 1_7 GR promoter contributes to the attenuation of GR expression in Low LG-ABN adult offspring, whereas increased NGFIA binding to the hyperacetylated and hypomethylated response element on the exon 1_7 GR promoter in the offspring of the High LG-ABN mothers would serve to maintain the differences in gene expression. DNA methylation represents a stable epigenetic mark; therefore, our findings provide an explanation for the enduring effect on mother-infant interactions over the first week of postnatal life on HPA responses to stress in the offspring.

Chapter 7
Reversal of Maternal Programming in Adulthood

The experimental procedures and analysed results described in this chapter were published in *The Journal of Neuroscience* (Weaver et al., 2005). Author contributions: I.C.G.W., M.J.M. and M.S. designed research; I.C.G.W. performed research; M.J.M. and M.S. contributed new reagents/analytic tools; I.C.G.W. analyzed data; and I.C.G.W., M.J.M. and M.S. wrote the paper.

The studies shown in Chapter 6 suggest that epigenetic mechanisms are involved in the long-term maternal programming of the offspring's responses to stress in adulthood. The exon 1_7 GR promoter sequence is uniquely expressed in the brain and exhibits considerable transactivational activity (McCormick et al., 2000). The adult offspring of Low LG-ABN mothers express significantly lower levels of the hippocampal exon 1_7 mRNA transcript in comparison to the offspring of High LG-ABN mothers. In the adult offspring of Low LG-ABN mothers the exon 1_7 GR promoter is hypermethylated, associated with hypoacetylated histone H3-K9 and reduced binding to the transcription factor NGFI-A, whereas in the adult offspring of High LG-ABN mothers the promoter is hypomethylated, associated with hyperacetylated histone H3-K9 and bound to increased levels of NGFI-A (see section 6.1). Central infusion of the HDAC inhibitor TSA enhanced histone H3-K9 acetylation of the exon 1_7 GR promoter in the offspring of the Low LG-ABN mothers, increased NGFI-A

binding, induced hypomethylation of CpG dinucleotide sequences in the promoter and eliminated the maternal effect on hippocampal GR expression and HPA responses to stress (see section 6.2). Although commonly thought that DNA methylation patterns are plastic during development and fixed in adult post-mitotic tissue, these data imply that demethylation machinery is present in the adult brain and can gain access to methylated genes by pharmacologically-induced alterations in chromatin conformation. If indeed the epigenetic status of the adult genome is truly in a dynamic equilibrium of methylation-verses-demethylation, it should be possible to reverse the DNA methylation pattern in the other direction by inducing hypermethylation.

Dietary methionine is converted by methionine adenosyl-transferase (MAT) into S-adenosyl-methionine (AdoMet, also termed SAM) (Cantoni, 1975; Mudd and Cantoni, 1958), which serves as the donor of methyl groups for DNA methylation. The methyl-donor SAM has been proposed to cause DNA hypermethylation by either activating DNA methylation enzymes or inhibiting active demethylation (Detich et al., 2003). Importantly, the synthesis of SAM is dependent on the local availability of methionine (Cooney, 1993). We reasoned that if the DNA methylation pattern of the exon 1_7 GR promoter within the adult offspring remains sensitive to both DNA methylation and DNA demethylation enzymes, then increasing brain methionine levels should result in DNA hypermethylation and a reversal of the maternal programming of GR expression and HPA responses to stress. Our current findings support this hypothesis, and suggest that the methylation status of specific DNA sequences is modifiable even in post-mitotic cells and thus the otherwise stable effects of maternal care on phenotype are potentially reversible.

7.1. *Experiment A*

The purpose of this experiment was to determine whether the DNA methylation pattern of the exon 1_7 GR promoter within the adult offspring remains sensitive to both DNA methylation and DNA demethylation enzymes, and if so, whether changes in promoter me-

thylation would be accompanied with changes in GR expression and hippocampal function in HPA responses to stress.

7.1.1. Animals and behaviour

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Catherine's, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. For further methodological details, see section 4.1.

7.1.2. Intracerebroventricular infusions

Same procedure as described before in section 4.3. In this study, a total volume of 2 µl of L-methionine (100 µg/ml in saline) or saline vehicle alone was injected (using a Hamilton 10 µl micro-syringe) through the infusion cannula over a 1-min period.

7.1.3. Sodium bisulfite mapping

Same procedure as described before in section 6.1.2.

7.1.4. 5-mC and NeuN immunohistochemistry

Same procedure as described before in section 4.8.

7.1.5. Chromatin immunoprecipitation (ChIP) assay

Same procedure as described before in section 6.1.3.

7.1.6. Semi-quantitative reverse transcription PCR (RT-PCR)

Same procedure as described before in section 4.13.

7.1.7. Western blotting analysis

Same procedure as described before in section 4.9.

7.1.8. HPA response to restraint stress

Same procedure as described before in section 4.6.

7.1.9. Forced-swim paradigm

Same procedure as described before in section 4.5.

7.1.10. Results

7.1.10.1. The effects of methionine on the methylation pattern of the exon 1_7 promoter

To determine the effects of methionine treatment, adult offspring of High and Low LG-ABN mothers were infused with methionine (100 μg/ml) or saline vehicle once a day for 7-consecutive-days and differences in methylation were mapped using the sodium bisulfite technique (Clark et al., 1994; Comb and Goodman, 1990), with particular interest in the NGFI-A consensus sequence (Figure 25a). Statistical analysis of the data across all 17 CpG sites (Figure 25b,c) revealed a significant effect of Group [$F(1,272) = 93.2$, $P < 0.0001$], Treatment [$F(1,272) = 52.8$, $P < 0.0001$] and Region [$F(16, 272) = 30.4$, $P < 0.0001$], as well as a significant Group x Treatment x Region interaction [$F(16,272) = 2.1$, $P = 0.0$], Group x Treatment interaction [$F(1,272) = 19.9$, $P < 0.0001$], Group x Region interaction [$F(16,272) = 4.1$, $P < 0.0001$] and Treatment x Region interaction [$F(16,272) = 2.8$, $P < 0.0001$]. The results revealed significant differences in the methylation of a number of regions of the exon 1_7 GR promoter sequence (Figure 25b) with significant differences within the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides of the NGFI-A consensus sequence (Figure 25c). As previously reported (Weaver et al., 2004), the cytosine within the 5′ CpG (site 16) dinucleotide is heavily methylated in the offspring of Low LG-ABN mothers, and rarely in the offspring of High LG-ABN mothers. In contrast, the cytosine within the 3′ CpG (site 17) dinucleotide of the NGFI-A consensus sequence is usually methylated, independent of maternal care. Statistical analysis of the data from these two sites revealed a highly significant effect of Group [$F(1,32) = 54.2$, $P < 0.0001$], Treatment [$F(1,32) = 74.3$, $P < 0.0001$] and Region [$F(1,32) = 115.3$, $P < 0.0001$], as well as a significant Group x Treatment interaction [$F(1,32) = 20.0$, $P < 0.0001$], Group x Region interaction [$F(1,32) = 47.2$, $P < 0.000$] and Treatment x Region interaction [$F(1,32) = 4.5$, P

= *0.04*]. *Post-hoc* analysis revealed that the cytosine within the 5′ CpG dinucleotide of the NGFI-A consensus sequence is significantly (*$P < 0.0001$) hypermethylated in the adult offspring of Low LG-ABN mothers compared to the offspring of High LG-ABN dams, and that methionine treatment significantly (**$P < 0.001$) increased cytosine methylation within the 5′ CpG dinucleotide in the offspring of High LG-ABN mothers in comparison to vehicle-treated High LG-ABN mothers. Thus, methionine treatment produced 'remethylation' of the 5′ CpG (site 16) dinucleotide in the offspring of High LG-ABN mothers (Figure 25c). These findings suggest that methionine treatment can reverse the hypomethylated status of the exon 1_7 GR promoter in the adult offspring of High LG-ABN mothers.

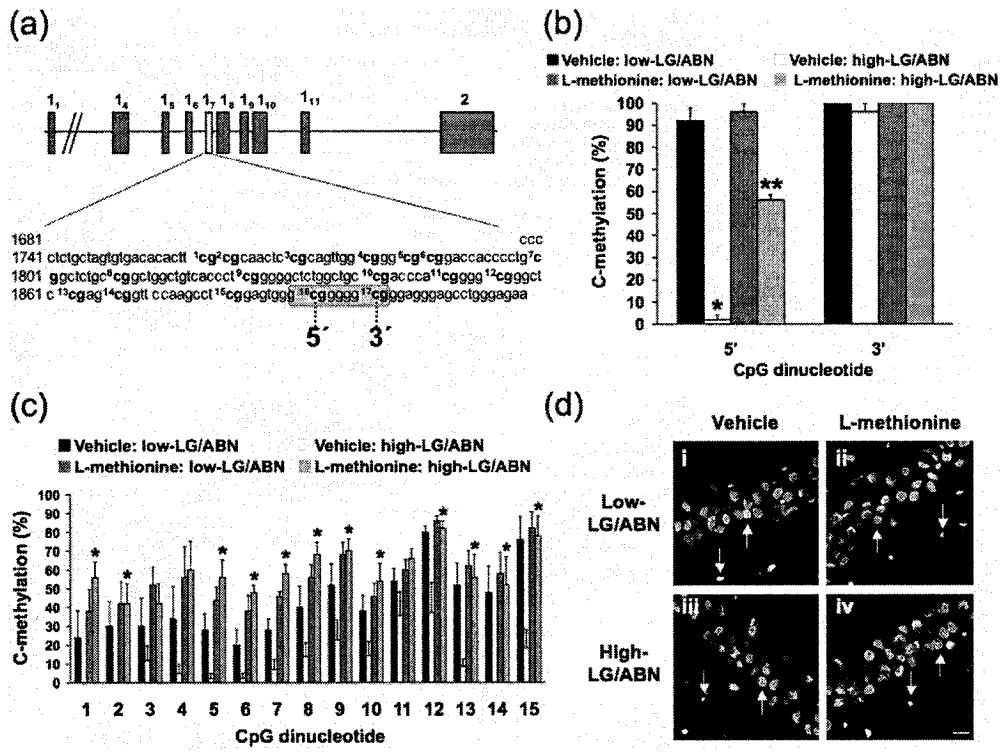

Figure 25. Methionine alters cytosine methylation of GR promoter. (**a**) Sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (bold) and the NGFI-A consensus sequence (McCormick et al., 2000) (encircled). (**b**,**c**) Methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from vehicle- and methionine-treated (100 μg/ml) adult High and Low LG-ABN offspring (6–10 clones sequenced/animal; n = 4 animals/group; *$P < 0.01$). (**b**) Percentage of methylated cytosine residues (mean ± SEM) for the first 15 CpG dinucleotides (*$P < 0.05$). (**c**) Percentage of methylated cytosine residues (mean ± SEM) for the 5′ (site 16) and 3′ (site 17) CpG dinucleotides within the NGFI-A consensus sequence (*$P < 0.0001$; **$P < 0.001$). (**d**; panel i-iv) Confocal photomicrographs of representative 5-mC-positive neurons located within the CA1 hippocampal region of Ammon's horn from vehicle- and methionine-treated (100 μg/ml) adult High and

Low LG-ABN offspring (n = 6 animals/group with nine sections/animal). Only large round nuclei corresponding to neuronal nuclei (indicated by arrows pointing upward) were included for analysis, partial or smaller nuclei (indicated by arrows pointing downward) were not included in the quantification. Scale bar, 50 μM.

Maternal care altered the methylation status of other CpG dinucleotides in the exon 1_7 sequence; in the case of sites 1, 2, 5, 6, 7, 8, 9, 10, 12, 13, 14 and 15, the methylation was similarly reversed with central methionine infusion (Figure 25b). The significance of these sites for transcription factor binding is currently unknown and a focus of ongoing studies. Thus, stable DNA methylation marking by maternal behaviour is reversible in the adult offspring hippocampus by increases in methionine. Methionine altered the methylation of the 5′ CpG (site 16) dinucleotide within the NGFI-A consensus sequence that is critical for the effect on NGFI-A binding to the exon 1_7 promoter.

To further examine whether maternal care or methionine treatment affected global DNA methylation levels, dorsal-hippocampal coronal sections from the methionine- or vehicle-treated adult offspring were co-immuno stained using antibodies specific for 5-methylcytosine (5-mC) or neuronal nuclei (NeuN) to assess genomic methylation levels. The tissue sections were analyzed thoroughly over the entire area of the dentate gyrus, and CA1, CA2 and CA3 hippocampal regions of Ammon's horn. However, the staining intensity within the different regions remained the same, regardless of maternal care or methionine-treatment. A representative image of 5-mC staining within the CA1 region from each treatment group is shown in Figure 25d (panel i-iv). Neuronal nuclei were only considered positive for 5-mC if they were round and intensely fluorescent in strong contrast to the surrounding tissue. 5-mC labelling intensity and background were consistent among slides. ANOVA revealed no significant effect of Group (vehicle treated High LG-ABN vs. vehicle treated Low LG-ABN [$F(1,16)= 0.53,\ p > 0.05$]) or Treatment (vehicle-treated Low LG-ABN vs. methionine-treated Low LG-ABN [$F(1,16) = 0.42,\ P > 0.05$]; vehicle-treated High LG-ABN vs. methionine-treated High LG-ABN [$F(1,16) = 0.37,\ P > 0.05$]). These results show that neither maternal care nor methionine treatment affected global DNA methylation levels. Our findings suggest that alterations of cytosine methylation in the adult brain through global procedures are surprisingly specific. Methionine alone does not methylate DNA, but is converted to the methyl donor SAM in the DNA methylation reaction. An increase in SAM would change the DNA methylation pattern of a gene only if the DNA

machinery was present on the gene. Since the DNA is not replicating it is not expected that maintenance DNA methyltransferases (DNMTs), which are normally present in the replication fork, would be ubiquitously present. It is becoming clear now that chromatin and DNA methylation enzymes are targeted to specific genes in a regulated process. Thus, the specificity of the effect of methionine is likely determined by the occupancy of distinct promoters by DNMTs. It is tempting to speculate that genes involved in crucial regulatory functions, such as GR, are persistently associated with the DNA methylation machinery and are thus hypersensitive to global changes in methionine levels. Future experiments will elucidate the mechanisms, which target the DNA methylation/demethylation machinery to specific genes such as GR in the hippocampus.

7.1.10.2. The effects of methionine on binding of NGFI-A to the exon 1_7 promoter

Differences in methylation of the exon 1_7 GR promoter are tightly associated with effects on histone acetylation and NGFI-A binding (Weaver et al., 2004). We tested the hypothesis that the effect of methionine on DNA methylation results in (i) decreased histone acetylation at the K9 residue of the H3 histone(s) associated with the exon 1_7 GR promoter, (ii) decreased interaction between NGFI-A and the promoter sequence or (iii) changes in both histone acetylation and NGFI-A association with the GR promoter. We performed a chromatin immunoprecipitation (ChIP) analysis of histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter in the native chromatin environment *in vivo*. Intact hippocampi from vehicle or methionine (100 μg/ml) treated offspring of High and Low LG-ABN mothers were cross-linked *in vivo* with para-formaldehyde perfusion. We then selectively immunoprecipitated protein-DNA complexes with an antibody against either acetylated histone H3-K9 or NGFI-A. The protein-DNA complexes were uncross-linked, and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence (Figure 26a-c). ANOVA revealed a significant Group effect [$F(1,16) = 10.0$, $P = 0.01$], but no significant Group x Treatment interaction effect for histone H3-K9 acetylation [$F(1,16) = 0.8$, $P > 0.01$]. However, there was a significant Group x Treatment interaction effect for NGFI-A [$F(1,16) = 7.9$, $P = 0.01$]. *Post-hoc* analysis showed that vehicle- and methionine-treated offspring of High LG-ABN mothers showed significantly ($*P < 0.01$) greater H3-K9 association with the

exon 1_7 sequence than any other group (Figure 26a,c), suggesting no effect of methionine treatment on H3-K9 acetylation. In contrast, vehicle-treated offspring of High LG-ABN mothers showed significantly (**$P < 0.001$) greater NGFI-A association with the exon 1_7 sequence than any other group (Figure 26b,c). These results indicate decreased binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the adult offspring of methionine-treated High LG-ABN mothers compared with the vehicle-treated offspring of High LG-ABN mothers (Figure 26b,c); there were no significant differences between methionine treated offspring of High LG-ABN mothers and either vehicle- or methionine-treated offspring of Low LG-ABN dams. The absence of any methionine effect on the offspring of Low LG-ABN mothers was expected since in these animals the exon 1_7 GR promoter region shows little NGFI-A binding.

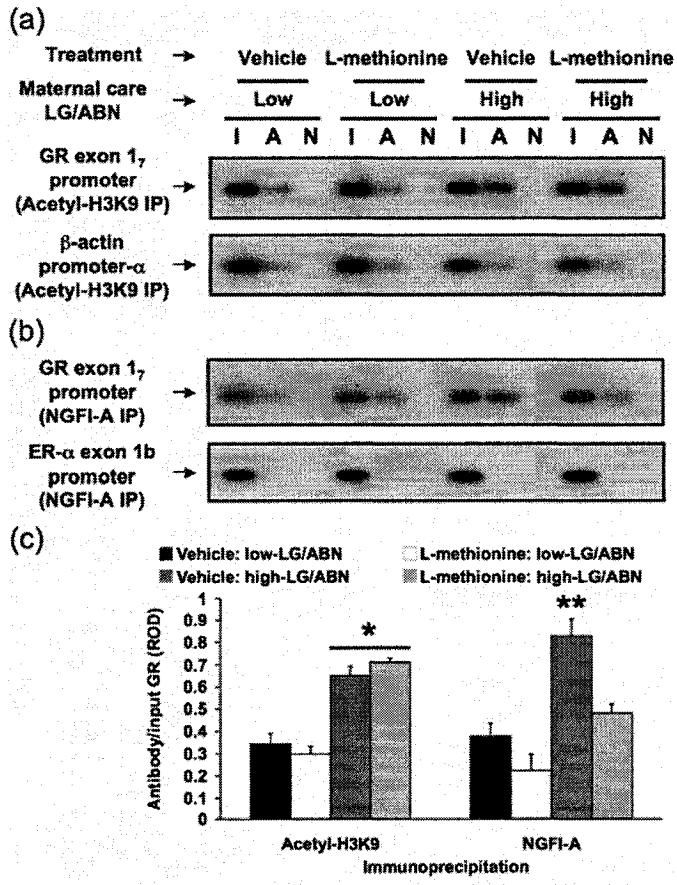

Figure 26. Methionine eliminates maternal effect on NGFI-A binding independently of histone acetylation. Chromatin immunoprecipitation analysis of the association between histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR promoter sequence in hippocampal tissue from vehicle- and methionine-treated (100 μg/ml) adult offspring of High and Low LG-ABN mothers (n = 4

animals/group). (**a**,**b**) Lanes were loaded with non-immunoprecipitated input (I), acetylated histone H3-K9 (top) or NGFI-A (middle) primary antibody immunoprecipitated (A), or non-immune IgG antibody immunoprecipitated (N) hippocampal extracts. (**a**) Representative Southern blot of the amplified exon 1_7 region from acetyl-histone H3-K9 immunoprecipitated hippocampal tissue (194 bp band). DNA loading was controlled using primers specific for the ubiquitously expressed β-actin promoter-α region (171 bp band). (**b**) Representative Southern blot of the amplified exon 1_7 region of the GR from NGFI-A immunoprecipitated hippocampal tissue (194 bp band). Exon 1b ER-α promoter region, which does not contain NGFI-A recognition elements (493 bp), amplified from the same NGFI-A immunoprecipitated hippocampal tissue was run as a control for specificity and showed no signal. (**c**) Relative optical density (ROD; mean ± SEM) of exon 1_7 sequence amplified from acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue from vehicle- and methionine-treated (100 μg/ml) adult High and Low LG-ABN offspring (n = 4 animals/group; *$P < 0.01$; **$P < 0.001$).

In summary, methionine treatment decreased binding of NGFI-A to the hippocampal exon 1_7 GR promoter, but did not affect levels of histone acetylation. DNA methylation is known to silence gene expression through two different mechanisms. Site-specific DNA methylation, such as methylation of the 5′ CpG dinucleotide within the NGFI-A binding site, which directly blocks binding of the transcription factor to its cognate sequence and thus inhibits transcription without necessarily altering chromatin structure. The second mechanism is indirect. Methylated DNA binding proteins, such as methyl-CpG binding protein (MeCP)-2, are recruited to a region of dense methylation and target HDACs to the chromatin resulting in histone deacetylation and subsequent chromatin inactivation (Jones et al., 1998; Nan et al., 1998). Though we cannot preclude the involvement of other acetylation sites within histone H3 or the involvement of acetylation sites within other histones (e.g., histone H4), our data are consistent with the hypothesis that the methionine-induced remethylation of the NGFI-A binding site inhibits transcription by the direct mechanism through blocking NGFI-A binding without altering histone acetylation. This mechanism is also consistent with previous data that revealed an absence of MeCP-2 binding within the hypermethylated GR exon 1_7 promoter region of the adult Low LG-ABN offspring. Thus, methylation in this case does not result in recruitment of methylated DNA binding proteins and histone deacetylation. We previously showed that though site-specific methylation is sufficient to block NGFI-A binding to the GR exon 1_7 promoter later in adulthood (as shown here), the GR promoter is both methylated and associated with deacetylated histones at postnatal day-1 (see Chapter 6). We propose that induction of histone acetylation between postnatal day-1 and 6 enables demethylation of the 5′ CpG dinucleotide and binding of NGFI-A resulting in transcription activation. Thus, there is a unidirectional causal rela-

tionship between the state of histone acetylation and the state of methylation of GR exon 1_7 promoter, whereby the state of methylation is determined by the state of histone acetylation and either developmental or pharmacological change in histone acetylation results in demethylation, but methylation does not necessarily alter histone acetylation. Thus, the increased methylation of the 5′ CpG site on the exon 1_7 promoter with methionine treatment may be sufficient to inhibit NGFI-A binding, even in the absence of changes in histone acetylation, through site-specific exclusion of transcription factor association.

7.1.10.3. Effects of methionine on GR expression

The effect of maternal care on HPA responses to stress is associated with differences in hippocampal GR gene expression and GC feedback sensitivity (Meaney, 2001). GR gene expression in the hippocampus is increased in the adult offspring of High compared with Low LG-ABN mothers (Francis et al., 1999). This difference is mediated by the differential methylation of the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence in the exon 1_7 GR promoter and the subsequent alteration of histone acetylation and NGFI-A binding to the exon 1_7 sequence (see Chapter 6). If the differential epigenetic marking regulates the expression of the exon 1_7 GR promoter in High versus Low LG-ABN offspring, then reversal of the epigenetic marking should accompany a decrease in hippocampal GR expression. To examine whether maternal LG-ABN behaviour or methionine treatment affected hippocampal exon 1_7 GR mRNA levels, RT-PCR analysis was performed using purified hippocampal mRNA from the vehicle- or methionine-treated adult offspring of High and Low LG-ABN mothers (Figure 27a). As a positive control, hippocampal mRNA from TSA-treated adult offspring of Low LG-ABN mothers was also analyzed since this manipulation has been previously shown to increase GR protein expression. ANOVA revealed a significant effect Group [$F(1,16) = 9.0$, $P = 0.01$] and Treatment [$F(1,16) = 8.0$, $P = 0.01$] and significant Group x Treatment interaction [$F(1,16) = 11.0$, $P > 0.05$]. *Post-hoc* analysis showed that TSA-treated offspring of Low LG-ABN mothers and vehicle-treated offspring of High LG-ABN dams showed significantly ($^*P < 0.001$ and

$^{**}P < 0.002$, respectively) greater exon 1_7 GR mRNA levels than any other group (Figure 27b), suggesting that methionine treatment significantly reduces hippocampal exon 1_7 GR mRNA levels in the offspring of High LG-ABN dams. This is further supported by the results (Figure 27c,d) showing that hippocampal GR protein expression was also significantly decreased in methionine-treated offspring of High LG-ABN mothers to levels that were comparable to those of either the vehicle- or methionine-treated offspring of Low LG-ABN mothers. ANOVA revealed highly significant main effects of Group [$F(1,16) = 8.2$, $P = 0.01$] and Treatment [$F(1,16) = 16.9$, $P < 0.0001$], as well as a significant Group x Treatment interaction effect [$F(1,16) = 4.2$, $P = 0.05$]. *Post-hoc* analysis indicated that methionine treatment significantly decreased hippocampal GR expression in the offspring of High LG-ABN mothers (vehicle-treated High LG-ABN offspring vs. methionine-treated High LG-ABN offspring, $^{*}P < 0.001$), eliminating the difference in hippocampal GR expression between the offspring of Low or High LG-ABN mothers (methionine-treated High LG-ABN offspring vs. methionine-treated Low LG-ABN offspring, $P > 0.90$). Although methionine treatment significantly reduced GR expression in High LG-ABN adult offspring, global abundance of protein in the hippocampus was not apparently decreased, as indicated by the equal α-tubulin immunoreactivity (Figure 27c).

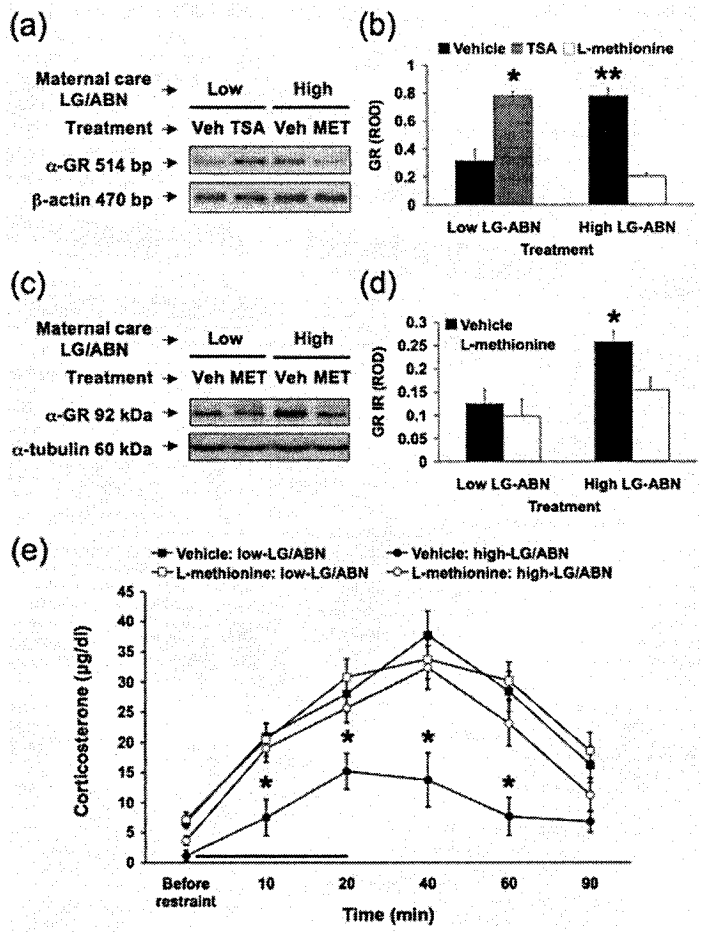

Figure 27. Methionine eliminates the maternal effect on hippocampal GR expression and HPA responses to stress. (**a**) Representative RT-PCR illustrating absolute levels of hippocampal GR exon 1_7 mRNA transcript levels from vehicle-, TSA (100 ng/ml)- and methionine (100 μg/ml)-treated adult offspring of High and Low LG-ABN mothers (n = 5 animals/group). Molecular weight markers (MBI Fermentas Life Sciences) correspond to a single major band at 514 bp. The lower panel shows the RT-PCR for β-actin, illustrating absolute hippocampal mRNA transcript levels. Molecular weight markers correspond to a single major band at 470 bp and the intensity of the signal was similar in all lanes. (**b**) Relative optical density (ROD; mean ± SEM) of hippocampal GR exon 1_7 mRNA transcript levels from vehicle-, TSA (100 ng/ml)- and methionine (100 μg/ml)-treated adult offspring of High and Low LG-ABN mothers (n = 5 animals/group; *$P < 0.002$, **$P < 0.001$). (**c**) Representative western blot illustrating absolute levels of electrophoresed hippocampal GR immunoreactivity (IR) from vehicle- and methionine-treated (100 μg/ml) adult offspring of High and Low LG-ABN mothers (n = 5 animals/group). Molecular weight markers (SeeBlue, Santa Cruz Biotech) correspond to a single major band at 92 kDa. The lower panel shows the membrane reprobed for α-tubulin IR, illustrating absolute levels of electrophoresed hippocampal protein bound to the transfer membrane. Molecular weight markers correspond to a single major band at ~60 kDa and the intensity of the signal was similar in all lanes. (**d**) Relative optical density (ROD; mean ± SEM) of hippocampal GR IR levels from vehicle- and methionine-treated (100 μg/ml) adult offspring of High and Low LG-ABN mothers (n = 5 animals/group; *$P < 0.001$). (**e**) Mean ± SEM plasma corticosterone responses to a 20-min period of restraint stress (solid bar) in vehicle- and methionine-treated (100 μg/ml) adult offspring of High and Low LG-ABN mothers (n = 10 animals/group; *$P < 0.01$).

7.1.10.4. Effects of methionine on HPA responses to stress

As adults, the offspring of High LG-ABN mothers show increased hippocampal GR expression, enhanced GC feedback sensitivity and more modest HPA responses to stress than the offspring of Low LG-ABN mothers (Liu et al., 1997). Given that methionine treatment reversed the group difference in hippocampal GR expression, we examined the adrenocortical responses to stress in a separate cohort of vehicle- and methionine-treated animals (Figure 27e). Statistical analysis of the plasma corticosterone data revealed significant effects of Group [$F(1,34) = 4.3$, $P = 0.05$], Treatment [$F(1,34) = 5.2$, $P = 0.05$] and Time [$F(1,34) = 22.4$, $P < 0.0001$], as well as a significant Group x Treatment interaction effect [$F(1,34) = 7.8$, $P = 0.001$]. *Post-hoc* analysis revealed that plasma corticosterone responses to restraint stress in the vehicle-treated adult offspring of High LG-ABN mothers were significantly ($*P < 0.01$) lower than those of methionine- and vehicle-treated adult offspring of Low LG-ABN mothers or methionine-treated offspring of High LG-ABN mothers. The HPA response to stress in the offspring of Low LG-ABN mothers was unaffected by methionine treatment.

7.1.10.5. The effects of GR on behavioural responses to stress

Rodents forced to swim in a restricted space from which they cannot escape eventually cease apparent attempts to escape and become immobile (Porsolt et al., 1977). Such learned-helplessness-induced immobility is selectively sensitive to antidepressant treatments (Porsolt et al., 1978). While adrenalectomized rats acquire the immobile response normally, they are unable to retain the response in subsequent testing and the effects are reversed by GC administration (Funder, 1989). Since maternal behaviour affects the level of circulating GCs and hippocampal GR expression in the offspring, we used the forced-swim test as a model to assess the effects of methionine treatment on behavioural responses to stress in the adult offspring of High and Low LG-ABN mothers (Figure 28). Statistical analysis of the length of time the animals spent immobile revealed significant effects of Group [$F(1,34) = 4.3$, $P = 0.048$], Treatment [$F(1,34) = 4.3$, $P = 0.046$] as well as a significant Group x Treatment interaction [$F(1,34) = 7.7$, $P = 0.009$]. *Post-hoc* analysis revealed

that methionine treatment significantly increased the length of time the offspring of High LG-ABN mothers spent immobile in the forced swim-test, comparable to the Low LG-ABN animals. Thus, the length of time the vehicle-treated adult offspring of High LG-ABN mothers spent immobile was significantly (*$P < 0.01$) shorter in comparison to the methionine- and vehicle-treated adult offspring of Low LG-ABN mothers or methionine-treated offspring of High LG-ABN mothers. Forced swim-test performance by the Low LG-ABN offspring was unaffected by methionine treatment. In summary, methionine treatment reversed endocrine and behavioural responses to stress in the adult offspring of High LG-ABN mothers as well as epigenetic programming of the exon 1_7 GR promoter.

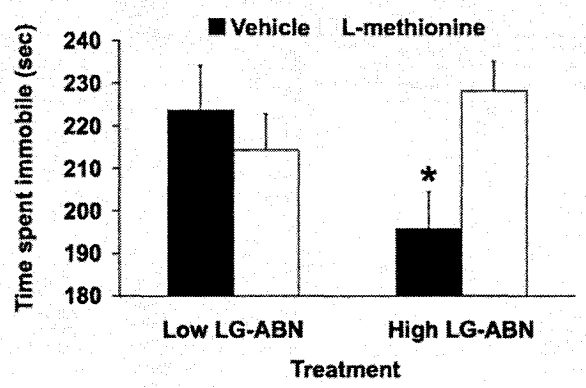

Figure 28. Methionine eliminates the maternal effect on behavioural responses to stress. Mean ± SEM time spent immobile during a 5-min period of forced-swim stress in vehicle- and methionine-treated (100 μg/ml) adult offspring of High and Low LG-ABN mothers (n = 10 animals/group; *$P < 0.01$).

7.2. *Discussion*

Methionine and epigenetic programming of stress responses by maternal behaviour

These studies reveal that methionine, a well established dietary modulator of macromolecule methylation, reverses both the DNA methylation pattern of GR promoter and stress responses, thus demonstrating a causal relationship between the programming of responses to stress by maternal behaviour and the epigenomic status of the adult offspring. The current data demonstrate that while epigenomic marks involving stable covalent modifications of the DNA triggered by maternal behaviour are stable through life, these marks are potentially reversible even in terminally differentiated tissues such as the hippocampus. Methy-

lation patterns are altered by inhibition of DNMTs or upon a change in the supply of methyl-donors in cell division during synthesis of the unmethylated nascent strand. However, it is generally believed that methylation patterns remain stable in post-mitotic tissues. Our findings provide novel support for a model that suggests a more dynamic process where methylation status is modifiable even in post-mitotic cells. These studies, together with the findings in Chapter 6 showing demethylation following chronic histone deacetylase blockade, demonstrate that the enzymatic machinery necessary for both demethylation and remethylation are operative in differentiated cells such as neurons.

Our previous studies in Chapter 6 demonstrate that, maternal behaviour alters the histone conformation and methylation status of the 5' CpG (site 16) dinucleotide within the NGFI-A consensus sequence, and the binding of NGFI-A to its cognate sequence *in vivo*. These data provide an epigenetic mechanism for long-term programming of stress responsivity by experience early in life. Moreover, pharmacological inhibition of histone deacetylation by TSA results in demethylation of the exon 1_7 GR promoter in the adult hippocampus and reversal of the effects of Low maternal LG-ABN on stress responsivity (see section 6.2). The data presented here show that methionine infusion causes remethylation of the exon 1_7 GR promoter and reversal of High maternal LG-ABN on stress responsivity, by a mechanism independent of histone H3-K9 acetylation. Considering that both TSA and methionine alter DNA methylation indirectly by challenging the accessibility and activity of DNMTs and demethylases, our findings demonstrate that the DNA methylation and demethylation machinery is present in the adult hippocampus and suggest that the balance of these activities plays a pivotal role in the stability of the DNA methylation pattern.

While the experiments presented here involved infusion of methionine into the left lateral ventricles, they raise the possibility that diet can affect the phenotypes being studied. Since intracellular levels of methionine seem to be affected by both dietary intake and polymorphisms of enzymes involved in methionine metabolism, such as methylenetetrahydrofolate-reductase (MTHFR) (Friso et al., 2002), it is tempting to consider, together with

our data, the possibility that diet could modify epigenetic programming in the brain not only during early development but also in adult life. Human epidemiologic and animal model data indicate that susceptibility to adult-onset chronic disease is influenced by persistent adaptations to prenatal and early postnatal nutrition (Lucas, 1998). Dietary L-methionine is crucial for normal brain development, brain aging and the pathogenesis of neurodegenerative disorders; playing an essential role in gene expression, protein synthesis, cell signalling, lipid transport/metabolism and neuron survival (Slyshenkov et al., 2002; Van den Veyver, 2002). DNA methyltransferase (MTase) requires SAM to establish or maintain 5-mC patterns. Synthesis of SAM is dependent on the availability of dietary folates, vitamin B_{12}, methionine, choline and betaine (Cooney, 1993). Maternal methyl supplements affect epigenetic variation and DNA methylation and positively affect health and longevity of the offspring (Cooney et al., 2002; Waterland and Jirtle, 2003; Wolff et al., 1998; Wylie et al., 2003). This could also have important therapeutic implications, since aberrant DNA methylation is involved in neurological disease such as fragile X syndrome and is potentially associated with multiple psychiatric and behavioural conditions including schizophrenia (Grayson et al., 2005). We hypothesize that reversal of epigenetic states in the brain, such as the remethylation of the exon 1_7 GR promoter illustrated here, could be triggered not only by pharmacological agents, but also by stable variations in environmental conditions.

Concluding remarks

In summary, I have shown that methionine, a well established dietary modulator of macromolecule methylation, reverses both the DNA methylation pattern of GR promoter and stress responses, thus demonstrating a causal relationship between the programming of responses to stress by maternal behaviour and the epigenomic status of the adult offspring. The current data demonstrate that while epigenomic marks involving stable covalent modifications of the DNA triggered by maternal behaviour are stable through life, these marks are potentially reversible even in terminally differentiated tissues such as the hippocampus. These findings provide novel support for a model that suggests a more dynamic process where methylation status is modifiable even in post-mitotic cells. These studies, together

with the findings in Chapter 6 showing demethylation following chronic histone deacetylase blockade, demonstrate that the enzymatic machinery necessary for both demethylation and remethylation are operative in differentiated cells such as neurons.

Chapter 8
Maternal Programming of the Hippocampal Transcriptome

The experimental procedures and analysed results described in this chapter were published in *Proceedings of the National Academy of Sciences of the United States of America* (Weaver et al., 2006). Author contributions: I.C.G.W., M.J.M. and M.S. designed research; I.C.G.W. performed research; M.J.M. and M.S. contributed new reagents/analytic tools; I.C.G.W. analyzed data; and I.C.G.W., M.J.M. and M.S. wrote the paper.

The studies shown in Chapter 6 and Chapter 7 suggest that maternal programming of individual differences in gene expression and stress responses in the rat involves modifications of epigenetic mechanisms including DNA methylation and histone modification of the NGFI-A transcription factor binding site within a brain-specific GR promoter. Increased maternal LG-ABN behaviour during the first week of life causes DNA demethylation, increased histone acetylation and NGFI-A binding and increased hippocampal GR expression (McCormick et al., 2000). Accordingly, the NGFI-A binding site within the hippocampal GR promoter is methylated and hypoacetylated in offspring of Low LG-ABN and demethylated and hyperacetylated in offspring of High LG-ABN (see section 6.1). However, central infusion of the HDAC inhibitor TSA eliminated the maternal effect on histone acetylation, DNA methylation, hippocampal GR expression and HPA responses to stress in the adult offspring of Low LG-ABN dams (see section 6.2). In contrast, central infusion of the

adult offspring of High LG-ABN mothers with the essential amino acid L-methionine, a precursor to S-adenosyl-methionine (SAM, also termed AdoMet) that serves as the donor of methyl-groups, resulted in increased methylation of the NGFI-A binding site in the hippocampal GR promoter, decreased GR expression and increased HPA responses to stress (shown in Chapter 7).

8.1. *Experiment A*

In this experiment, we reasoned that it remained unclear whether the effects of maternal care are limited to a single gene or whether they result in a global long-term reprogramming of the adult hippocampal transcriptome. Moreover, it remained unclear whether the expression of a maternally-regulated gene would be affected by the same pharmacological manipulations that reverse the effects of maternal care on GR expression. To address these questions, we used microarray analysis to examine the effects of maternal care on global gene expression within the hippocampal transcriptome of the adult offspring.

8.1.1. Animals and behaviour

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Catherine's, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. For further methodological details, see section 4.1.

8.1.2. Intracerebroventricular infusions

Same procedure as described before in section 4.3. In this study, a total volume of 2 µl of TSA (100 ng/ml in saline), L-methionine (100 µg/ml in saline) or saline vehicle alone was injected (using a Hamilton 10 µl micro-syringe) through the infusion cannula over a 1-min period.

8.1.3. Open-field paradigm

Same procedure as described before in section 4.4.

8.1.4. Affymetrix microarray analysis

Same procedure as described before in section 4.12.

8.1.5. Quantitative reverse transcription real-time PCR (QRT-PCR)

Same procedure as described before in section 4.14.

8.1.6. Results

8.1.6.1. Maternal effects on open-field behaviour and its reversal by TSA and methionine

Temporal lobe-kindled rats express lower levels of hippocampal GR and are behaviourally more anxious in an open-field arena (Kalynchuk and Meaney, 2003), suggesting that hippocampal gene expression may play a role in the development of anxiety-mediated behaviour. In the rat, ICV infusion of TSA or methionine can eliminate the maternal effect on hippocampal GR expression and endocrine and behavioural responses to stress (Chapter 6 and Chapter 7). Here we examined the effects of TSA and methionine on anxiety-induced behaviour in the open-field. The PND 90 (adult) male offspring of High and Low LG-ABN mothers were infused with saline vehicle, TSA (100 ng/ml) or methionine (100 μg/ml) once a day for 7 consecutive-days and 7 days after the final infusion the tested in an unfamiliar open-field arena (Figure 29a,b). Previous studies have shown that the behavioural effects of the TSA and methionine infusion are sustained for at least two weeks following the final infusion (Weaver et al., unpublished data.). Group differences as a function of maternal care were observed in the length of time the animals spent exploring in the inner-field, the critical measure of fear-behaviour (Figure 29a), although no differences were observed in overall activity (Figure 29b). ANOVA revealed a main effect of Group [$F(1,68) = 13.86$, $P < 0.001$] and Treatment [$F(2,68) = 10.44$, $P < 0.01$] and a Group x Treatment interaction effect [$F(2,68) = 12.96$, $P < 0.001$]. *Post-hoc* analysis revealed that the vehicle-treated offspring of Low-LG-ABN mothers spent significantly ($^{*}P < 0.001$) less time exploring in the inner-field in comparison to the vehicle-treated offspring of High LG-ABN dams. These effects are reversed by TSA and methionine treatment, respectively. TSA-treated

adult offspring of Low LG-ABN mothers spent significantly (**$P < 0.001$) more time exploring in the inner-field in comparison to the vehicle- and methionine-treated offspring of Low LG-ABN dams, whereas the methionine-treated adult offspring of High LG-ABN mothers spent significantly (***$P < 0.001$) less time in the inner-field in comparison to the vehicle- and TSA-treated offspring of High LG-ABN dams (Figure 1a). No significant differences were found between the lengths of time the TSA-treated High LG-ABN offspring or methionine-treated Low LG-ABN offspring spent exploring in the inner-field in comparison to their respective vehicle-treated control groups (TSA-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams; methionine-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams). These findings suggest that anxiety-induced behavioural responses programmed early in life are potentially reversible in adult life through manipulations known to alter DNA methylation.

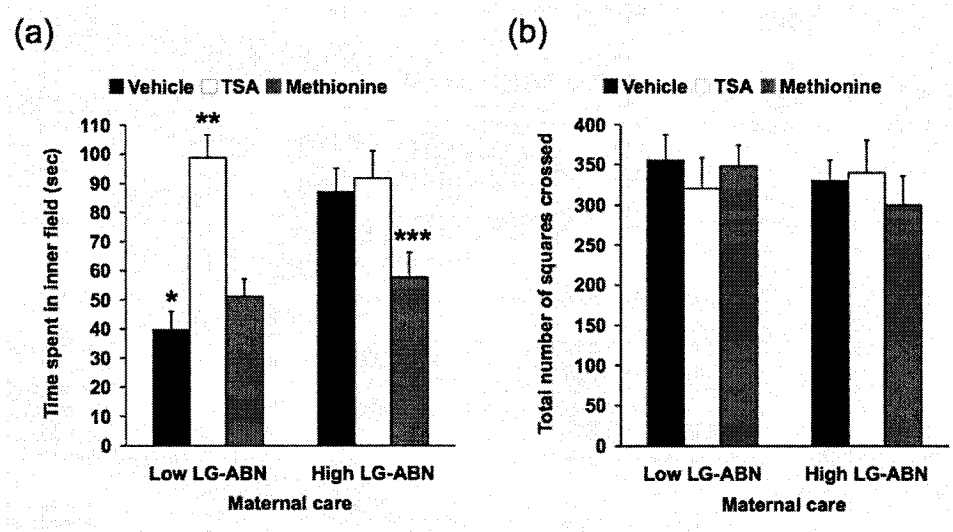

Figure 29. TSA and Methionine eliminate the maternal effect on behavioural responses to novelty stress. Open-field behaviour of TSA (100 ng/ml)-, methionine (100 µg/ml)- and vehicle-treated adult offspring of High and Low LG-ABN mothers during a 10-minute testing session (n = 10 animals/group). (**a**) Mean ± SEM time spent exploring in the inner-arena. *($P < 0.001$), vehicle-treated adult offspring of Low LG-ABN mothers Vs. vehicle-treated offspring of High LG-ABN dams; **($P < 0.001$), TSA-treated adult offspring of Low LG-ABN mothers Vs. vehicle- and methionine-treated offspring of Low LG-ABN dams; ***($P < 0.001$), methionine-treated adult offspring of High LG-ABN mothers Vs. vehicle- and TSA-treated offspring of High LG-ABN dams. (**b**) Mean ± SEM activity in the arena (n = 10 animals/group).

8.1.6.2. TSA and methionine effects on differential gene expression in the hippocampus

These long-term effects on behaviour are predicted to result from long-term stable reprogramming of gene expression profiles. To determine the influence on the hippocampal transcriptome, the vehicle and TSA-treated offspring of Low LG-ABN mothers, and the vehicle and methionine-treated offspring of High LG-ABN dams were sacrificed and affymetrix microarrays were employed to monitor changes in hippocampal expression of 31,099 unique mRNA transcripts. The four different treatment groups were compared to their respective control groups: (i) vehicle-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams, (ii) TSA-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams, (iii) methionine-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams. Transcripts with similar profiles within the same treatment group (i.e., same direction of expression) were averaged and only transcripts with mean expression levels significantly (Student t-test, $p < 0.05$) altered ≥ 1.5-fold between ≥ 1 group were included in subsequent analysis. In total, 935 different transcripts were present at significantly ($p < 0.05$) different levels between the three groups, representing 3 % of the total number of transcripts analyzed, while 97 % of the transcripts remained unaltered. Similarity in transcript expression levels between the four treatment groups was visualized by hierarchical clustering. Only those genes with a known biological function were included in the analysis. Accordingly, genes with unknown functions or classed as ESTs were excluded, leaving 100 well-characterized genes for two-way hierarchal clustering (Figure 30). The clustering analysis shows that 16 transcripts (45.71 % of the 35 maternal-responsive genes) were altered by maternal care alone, 31 transcripts (54.39 % of the 57 TSA-responsive genes) were altered by TSA treatment alone and 22 transcripts (53.66 % of the 41 methionine-responsive genes) were altered by methionine treatment alone. Thus, approximately half of those genes affected within each treatment group shown in Figure 30 were uniquely responsive to either maternal care, TSA or methionine treatment.

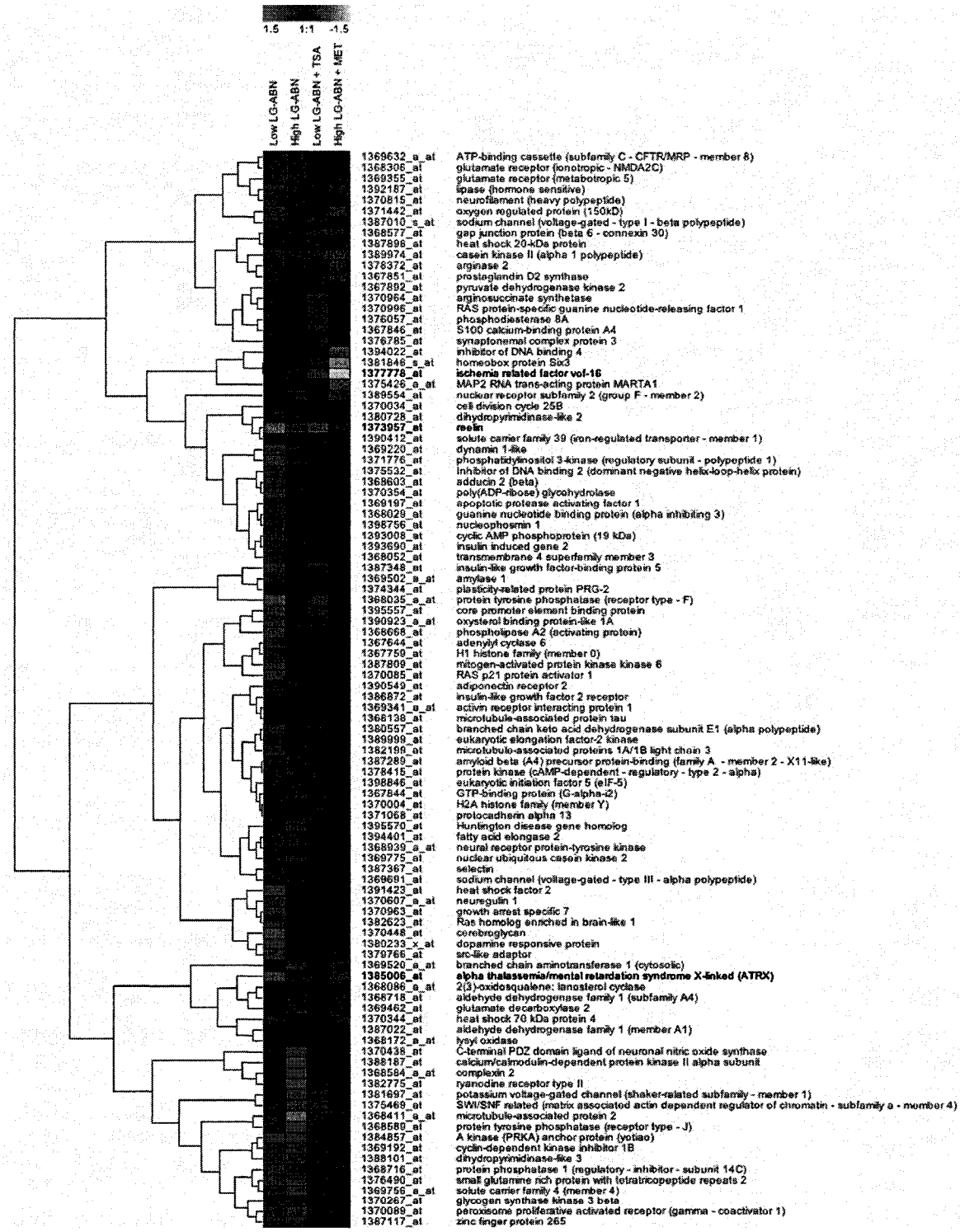

Figure 30. Two-way hierarchal clustering of 100 gene probe sets significantly altered in hippocampal tissue from TSA (100 ng/ml)-, methionine (100 µg/ml)- and vehicle-treated adult offspring of High and Low LG-ABN mothers (n = 3 animals/group). Gene probes are represented by rows with corresponding unique identity numbers, and columns represent experimental animals (vehicle-, TSA- and L-methionine-treated Low and High LG-ABN offspring). Shaded squares represent the average increased (dark) or decreased expression (light) for a single gene in each treatment group. Brightness is proportional to the magnitude of expression difference.

The dendogram also reveals that 12 transcripts were altered by both maternal care and TSA treatment, 5 transcripts were altered by both maternal care and methionine treatment, 12 transcripts were altered by both TSA and methionine treatment, whereas only 2 transcripts were altered by maternal care, TSA and methionine treatment. Together these results illustrate that the effects of maternal care, TSA and methionine treatment on the hippocampal transcriptome are gene specific.

8.1.6.3. TSA and methionine effects on gene activation and repression.

To examine the influence on the direction of gene expression, the four different treatment groups were again compared to their respective control groups as described above. The number of transcripts altered in the offspring of High LG-ABN mothers (vehicle-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) revealed that 253 transcripts (0.81 %) were up-regulated and 50 transcripts (0.16 %) were down-regulated, representing 0.97 % (n=303) of the total number of transcripts analyzed, while 99.03 % (n=30796) of the transcripts remained unaltered (Figure 31a). Thus, only a few hundred genes showed differences in expression in the adult offspring of High and Low LG-ABN mothers. Comparison of the number of transcripts altered in the TSA-treated offspring of Low LG-ABN mothers (TSA-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) revealed that 501 transcripts (1.61 %) were up-regulated and 42 transcripts (0.14 %) were down-regulated, representing 1.75 % (n=543) of the total number of transcripts analyzed, while 98.25 % (n=30556) of the transcripts remained unaltered (Figure 31b). Comparison of the number of transcripts altered in the methionine-treated offspring of High LG-ABN mothers (methionine-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams) revealed that 120 transcripts (0.39 %) were up-regulated and 217 transcripts (0.70 %) were down-regulated, representing 1.08 % (n=337) of the total number of transcripts analyzed, while 98.92 % (n=30762) of the transcripts remained unaltered (Figure 31c). The number of transcripts up-regulated in the TSA-treated offspring of Low LG-ABN mothers (TSA-

treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) was significantly ($t = 42.29$, $p < 0.001$) greater (~2-fold) in comparison to the number up-regulated in vehicle-treated offspring of High LG-ABN dams (vehicle-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) (Figure 31d, left-panel). Thus, TSA has a broader impact on gene expression than early life maternal care, which is expected since TSA is a global inhibitor of histone deacetylases. As anticipated from a manipulation that could increase DNA methylation that is known to result in gene silencing, the number of transcripts down-regulated in methionine-treated offspring of High LG-ABN mothers (methionine-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams) was significantly ($t = 35.94$, $p < 0.01$) greater in comparison to the number of transcripts down-regulated in the vehicle-treated offspring of Low LG-ABN dams (vehicle-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams) (Figure 31d, right-panel). Together, the total number of transcripts up-regulated in the vehicle-treated offspring of High LG-ABN mothers (vehicle-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) and TSA-treated offspring of Low LG-ABN mothers (TSA-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams), was significantly ($t = 51.87$, $P > 0.001$) greater (~2-fold) in comparison to the number of transcripts down-regulated in the vehicle-treated offspring of Low LG-ABN mothers (vehicle-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams) and methionine-treated offspring of High LG-ABN dams (methionine-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams).

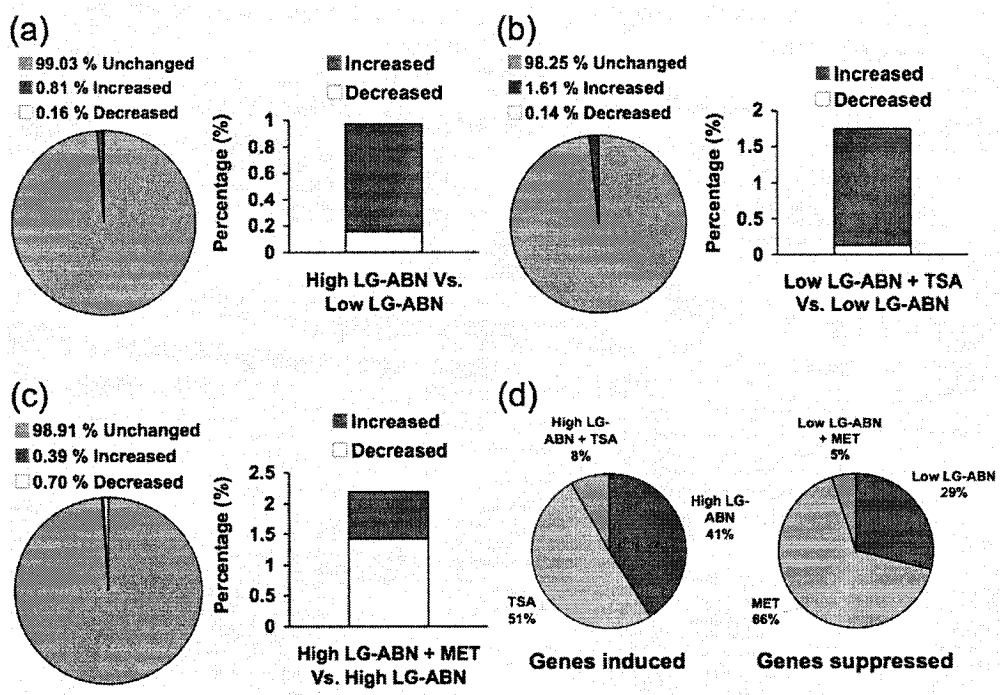

Figure 31. Direction of gene expression in hippocampal tissue from TSA (100 ng/ml)-, methionine (100 µg/ml)- and vehicle-treated adult offspring of High and Low LG-ABN mothers (n = 3 animals/group). (**a**) Percentage of mRNA transcripts increased, decreased or unchanged by High LG-ABN. (**b**) Percentage of mRNA transcripts increased, decreased or unchanged by TSA treatment. (**c**) Percentage of mRNA transcripts increased, decreased or unchanged by methionine treatment. (**d**) Percentage of mRNA transcripts increased by High LG-ABN, TSA treatment or both (left panel), and the percentage of mRNA transcripts decreased by Low LG-ABN, methionine treatment or both (right panel).

These data suggest first that maternal care during early life programs the expression of hundreds of genes in the adult offspring. Second, since the differences in gene expression are maintained well after the stimulus has gone, an epigenetic reprogramming of these genes might take place in response to maternal care to maintain their differential expression into adulthood. Third, the fact that the expression of a number of genes, which were normally programmed by maternal care were induced in Low-LG-ABN offspring by TSA or repressed in the offspring of High LG-ABN offspring by methionine suggests that the effect of early-life experience on the hippocampal transcriptome is potentially reversible in adulthood. Fourth, our analysis reveals the general direction of these changes in gene expression. Whereas High LG-ABN mainly affects stimulation of gene expression in com-

parison with Low LG-ABN and as expected TSA treatment results predominantly in activation of gene expression, methionine treatment primarily silences gene expression as expected from the general silencing effect of DNA hypermethylation.

8.1.6.4. Cellular functions of genes affected by maternal care.

The maternal care-, TSA- and methionine-regulated RNA transcripts are involved in several different classes of cellular function. A distinct class of regulated transcripts are derived from genes encoding protein products involved in general cellular metabolism and energy production that include several glycolytic enzymes, ATPases, ATP synthases and enzymes involved in lipid metabolism and mitochondrial components. A second class of molecules is comprised of factors involved in signal transduction pathways and includes membrane-bound receptors, intracellular messenger molecules, kinases, phosphatases and transcription factors. A third main class of regulated transcripts consists of predominantly ribosomal proteins, but also nucleolar proteins, ER-localized proteins and lysosomal membrane glycoproteins that are involved in protein synthesis, protein turnover, protein folding and intracellular trafficking of proteins. Finally a class of transcripts with diverse functions in the control of neuronal structure including cell adhesion molecules involved in neuronal connectivity, structural cellular components and synaptic components.

The most striking observation is that the maternal care-, TSA- and methionine-regulated RNA transcripts are all involved in similar classes of cellular function (Figure 32). However, when examined across all the categories, the distribution of genes up-regulated in the offspring of TSA-treated Low LG-ABN mothers is significantly different in comparison to the distribution of genes up-regulated in the offspring of vehicle-treated High LG-ABN dams ($\chi^2 = 12.425$, $p = 0.053$). Thus, though both TSA treatment and High maternal LG behaviour affected genes from similar categories, TSA treatment induced expression of a collection of unique transcripts that were different to those that were induced

in the offspring of High LG-ABN mothers. Furthermore, the distribution of genes down-regulated in the offspring of methionine-treated High LG-ABN mothers is significantly different in comparison to the distribution of genes down-regulated in the offspring of vehicle-treated Low LG-ABN dams ($\chi^2 = 6.513$, $p = 0.026$). Thus, though both methionine treatment and Low maternal LG-ABN behaviour affected genes from similar categories, methionine treatment suppressed expression of a collection of unique transcripts that were different to those that were suppressed in the offspring of Low LG-ABN dams.

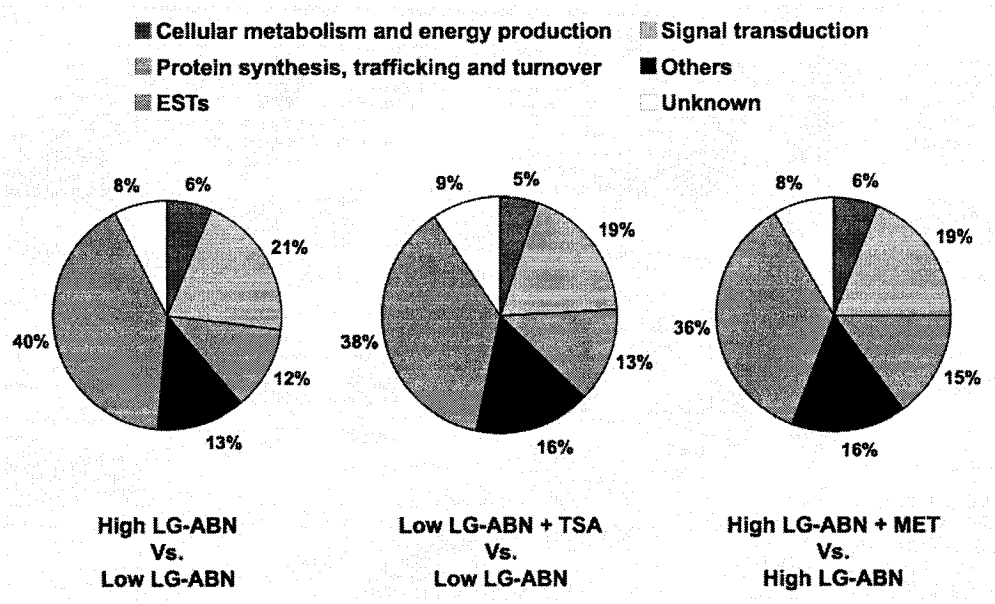

Figure 32. Distribution of maternal care-, TSA- and methionine-responsive genes over different functional classes.

Sequencing the genome of the rat (Rattus norvegicus) is a little behind that of the mouse and human. To date only 90% of the estimated 2.8 Gb genome is available (http://www.hgsc.bcm.tmc.edu/projects/rat/). Thus, it is difficult to determine, with great accuracy, the normal pattern of gene distribution from the entire rat genome. However, the pattern of the functional distribution of genes expressed is substantially different from the functional distribution of the 31,099 genes probe sets on the microarray. From the 31,099 unique probe sets on the rat 230.2 Affymetrix Genechip, 21 % encode protein products involved in general cellular metabolism and energy production, 12 % encode protein products

involved in signal transduction, 6 % encode proteins involved in protein synthesis, trafficking and turnover, 24 % encode protein products involved in cellular scaffolding, 3 % encode protein products with unknown functions and 34 % are EST tags. This suggests that the different treatments do have a specific effect on genes function with a known function.

To examine the effects of maternal care-, TSA- and methionine-treatment on RNA transcript levels within the different functional categories, the three treatment groups were compared to their respective control groups as previously described. Comparison of the transcripts altered in hippocampal tissue from offspring of vehicle-treated High LG-ABN mothers (vehicle-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) revealed that 19 transcripts (6 %) were involved in cellular metabolism and energy production, 63 transcripts (21 %) were involved in signal transduction and 36 transcripts (12 %) were involved in protein synthesis, trafficking and turnover (Figure 32; left panel). Comparison of the transcripts altered in hippocampal tissue from offspring of TSA-treated Low LG-ABN mothers (TSA-treated offspring of Low LG-ABN mothers *Vs.* vehicle-treated offspring of Low LG-ABN dams) revealed that 28 transcripts (5 %) were involved in cellular metabolism and energy production, 103 transcripts (19 %) were involved in signal transduction and 71 transcripts (13 %) were involved in protein synthesis, trafficking and turnover (Figure 32; middle panel). Comparison of the transcripts altered in hippocampal tissue from offspring of methionine-treated High LG-ABN mothers (methionine-treated offspring of High LG-ABN mothers *Vs.* vehicle-treated offspring of High LG-ABN dams) revealed that 21 transcripts (6 %) were involved in cellular metabolism and energy production, 63 transcripts (19 %) were involved in signal transduction and 51 transcripts (15 %) were involved in protein synthesis, trafficking and turnover (Figure 32; right panel). Together, these changes in mRNA expression demonstrate a clear effect of maternal care, TSA and methionine treatment on the adult hippocampal transcriptome.

8.1.6.5. Effects of TSA and methionine on ATRX, Reelin and Vof-16 gene expression

To validate the microarray data, three randomly chosen genes (shown in Figure 30) expressed within the different functional categories were subject to QRTPCR analysis. Predesigned primers were employed for amplifying the transcripts analyzed by the rat 230.2 GeneChip (Figure 33a-c). The results presented clearly show that for these three randomly chosen genes, the expression patterns obtained using the microarray chips are identical to those obtained using QRTPCR (compare the expression patterns in Figure 33a-c with those shown for these genes in Figure 30). For ATRX expression, ANOVA revealed a main effect of Group [$F(1,16) = 8.83$, $P < 0.05$] and Treatment [$F(1,16) = 7.29$, $P < 0.01$] and a main Group x Treatment interaction effect [$F(1,16) = 6.74$, $P < 0.05$]. *Post-hoc* analysis revealed that the level of ATRX expression was significantly ($*P < 0.001$) greater in the vehicle-treated offspring of High LG-ABN mothers in comparison to the vehicle-treated offspring of Low LG-ABN dams and methionine-treated offspring of High LG-ABN mothers (Figure 33a). The level of ATRX expression was also significantly ($**P < 0.01$) greater in the TSA-treated offspring of Low LG-ABN mothers in comparison to the vehicle-treated offspring of Low LG-ABN dams. No significant differences were found in the levels of ATRX expression between the vehicle-treated offspring of High LG-ABN mothers and TSA-treated offspring of Low LG-ABN dams, or the vehicle-treated offspring of Low LG-ABN mothers and methionine-treated offspring of High LG-ABN dams. For Reelin expression, ANOVA revealed main effects of Group [$F(1,16) = 8.76$, $P < 0.05$] and Treatment [$F(1,16) = 7.02$, $P < 0.01$] and a main Group x Treatment interaction effect [$F(1,16) = 5.73$, $P < 0.05$]. *Post-hoc* analysis revealed that the level of Reelin expression was significantly ($*P < 0.05$) greater in the vehicle-treated offspring of High LG-ABN mothers in comparison to any other group. The level of Reelin expression was also significantly ($**P < 0.01$) greater in the methionine-treated offspring of High LG-ABN mothers in comparison to the vehicle-treated offspring of Low LG-ABN mothers (Figure 33b). No significant differences in the levels of Reelin expression were found between the vehicle-

and TSA-treated offspring of Low LG-ABN dams, or the TSA-treated offspring of Low LG-ABN dams and methionine-treated offspring of High LG-ABN mothers. For Vof-16 expression, ANOVA revealed main effects of Group [$F(1,16) = 9.03$, $P < 0.05$] and Treatment [$F(1,16) = 6.72$, $P < 0.01$] and a main Group x Treatment interaction effect [$F(1,16) = 4.82$, $P < 0.05$]. *Post-hoc* analysis revealed that the level of Vof-16 expression was significantly ($*P < 0.01$) greater in the TSA-treated offspring of Low LG-ABN mothers in comparison to vehicle-treated offspring of Low LG-ABN dams. The level of Vof-16 expression was significantly ($**P < 0.001$) lower in the methionine-treated offspring of High LG-ABN mothers in comparison to any other group (Figure 33c). No significant differences in the level of Vof-16 expression were found between the vehicle-treated offspring of High and Low LG-ABN dams, or the vehicle-treated offspring of High LG-ABN mothers and TSA-treated offspring of Low LG-ABN dams. In summary, High licking and grooming increased hippocampal ATRX and Reelin expression with no significant effect on Vof-16 expression; TSA treatment increased hippocampal ATRX and Vof-16 gene expression with no significant effect on Reelin expression; whereas, Low LG-ABN and methionine treatment decreased expression of all three genes. The results suggest that the genes are indeed regulated by one or more treatment, underscoring the validity of the microarray data.

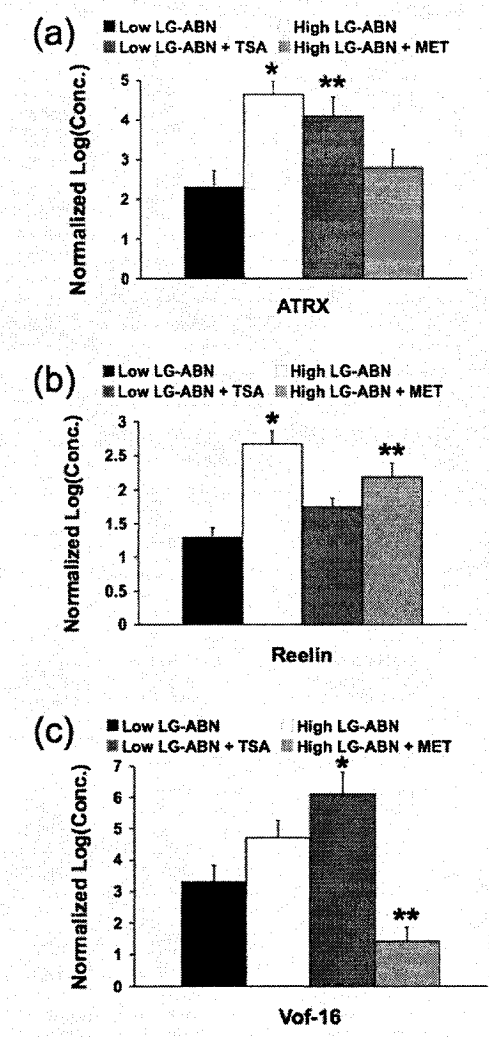

Figure 33. Methionine and TSA treatment eliminates the maternal effect on genes expressed within different functional classes. QRTPCR analysis of hippocampal gene expression in TSA (100 ng/ml)-, methionine (100 µg/ml)- and vehicle-treated adult offspring of High and Low LG-ABN mothers (n = 3 animals/group). (**a**) Mean ± SEM ATRX expression. *(*P < 0.001*), vehicle-treated offspring of High LG-ABN mothers Vs. vehicle-treated offspring of Low LG-ABN dams and methionine-treated offspring of High LG-ABN mothers; **(*P < 0.01*), TSA-treated offspring of Low LG-ABN mothers Vs. vehicle-treated offspring of Low LG-ABN dams. (**b**) Mean ± SEM Reelin expression. *(*P < 0.05*), vehicle-treated offspring of High LG-ABN mothers Vs. any other group; **(P < 0.01), methionine-treated offspring of High LG-ABN mothers Vs. vehicle-treated offspring of Low LG-ABN mothers. (**c**) Mean ± SEM Vof-16 expression. *(*P < 0.01*), TSA-treated offspring of Low LG-ABN mothers Vs. vehicle-treated offspring of Low LG-ABN dams; **(*P < 0.001*), methionine-treated offspring of High LG-ABN mothers Vs. any other group.

8.2. *Discussion*

TSA, methionine and programming of the hippocampal transcriptome by maternal behaviour

We have shown that gene expression is significantly altered in the hippocampus of adult rats as a function of maternal care early in life. In general, there were four major categories of affected genes. In all cases, expression was greater in the offspring of High compared to Low LG-ABN mothers. The genes were associated with: 1) cellular metabolism and energy production that include several glycolytic enzymes, ATPases, ATP synthases, enzymes involved in lipid metabolism and mitochondrial components; 2) Signalling molecules, receptors, and signal transduction proteins involved in the pathways that regulate brain formation and function (GABA, glutamate, somatostatin, dopamine); 3) ribosomal proteins and nucleolar proteins involved in protein synthesis, protein turnover, protein folding and intracellular trafficking of proteins; and 4) neuronal development, as well as extracellular matrix proteins and other structural molecules that define the architecture of the synaptic connections in the brain (cytoskeletal proteins). These differences in gene expression may, at least in part, form the molecular basis for the effect of early-life maternal care on the development HPA responses to stress in the offspring that are endured throughout life.

The mechanism responsible for the long-term programming of these genes by maternal care remains to be determined by future experiments. Nevertheless, based on our previous studies with the GR gene (see Chapter 6 and Chapter 7), we would like to propose that epigenetic reprogramming in response to maternal care is involved in the observed effects on gene expression. A signalling pathway triggered by maternal LG-ABN behaviour induces changes in chromatin structure and DNA modification, which then maintains this differential expression profile. The proposal that histone acetylation is involved is supported by the fact that some of the genes affected by maternal care are also induced by the HDAC inhibitor TSA. The fact that the methyl-donor L-methionine inhibits some of

the genes induced by maternal High LG-ABN supports the involvement of either DNA or histone methylation. Interestingly, although the effects of maternal care, TSA and methionine involve a large number of genes, the process is exquisitely specific. The vast majority of the genome is not affected, suggesting that these treatments in spite of their global nature do not result in a general collapse of gene expression programming. Although the basis for this specificity remains unknown, this observation has important implications on the future therapeutic utility of these and similar treatments.

Concluding remarks

In summary, programs could be modified by genetic alterations, which are transmitted in the germ line. Genetic polymorphisms have attracted significant attention as a possible mechanism underlying inter-individual behavioural differences and pathologies (Moret, 2004). Our data illustrates a new mechanism by which widespread and stable life-long inter-individual variation in gene expression in the brain might emerge. This mechanism does not require germ-line transmission and could be elicited by natural variations in maternal behaviour during early life. The main difference between genetic and epigenetic variation is the potential for reversal with the appropriate manipulation as illustrated here. Our data demonstrate the profound affects that early-life environment might have on the functioning of the genome and its life-long consequences on behaviour into adulthood.

Chapter 9
NGFI-A Mediated Epigenetic Programming by Maternal Behaviour

The previous chapters established that maternal care results in reprogramming of the offspring epigenome at a GR gene promoter in the hippocampus as well as potentially other gene loci. Offspring of mothers that showed high levels of LG-ABN behaviour were found to have differences in DNA methylation, as compared to offspring of Low LG-ABN mothers. These differences emerged over the first week of life, were reversed with cross-fostering, persisted into adulthood and were associated with altered histone acetylation and NGFI-A binding to the GR promoter (Chapter 6). Although the differences in gene expression between offspring of High and Low LG-ABN mothers were stable, they were also reversible by certain pharmacological manipulations (Chapter 7 and Chapter 8). These studies raise three critical and fundamental questions that lie at the heart of our conception of the long-standing problem of gene-environment interaction. First, what is the mechanism responsible for translating the behaviour of a mother into chemical changes in DNA? Second, why are these changes reflected in stable life long changes in gene expression? Third, what are the possible mechanisms that might reverse this programming? In this chapter we tested the hypothesis that the transcription factor NGFI-A (whose occupancy of the GR promoter is increased in offspring of High LG-ABN mothers as demonstrated in previous chapters) plays a pivotal role in translating the behavioural induced signalling into discreet sequence specific changes in epigenomic programming.

The following previously published data led us to suspect that the transcription factor NGFI-A is a critical mediator not only of the expression of GR in the adult animal but also of its epigenetic programming early in life. *In vivo* and *in vitro* studies suggest that maternal LG-ABN behaviour increases GR gene expression in the offspring through increased 5-hydroxytryptamine (serotonin, 5-HT) activity at 5-HT_7 receptors, and the subsequent activation of cyclic adenosine 3′,5′ monophosphate (cAMP) and cAMP-dependent protein kinase A (PKA) activity (Laplante et al., 2002; Meaney et al., 1987; Meaney et al., 2000). Both the *in vitro* effects of 5-HT and the *in vivo* effects of maternal behaviour on GR mRNA transcript expression are accompanied by an increased hippocampal expression of the transcription factor NGFI-A. The 8-bromo-cAMP and 5-HT effects on NGFI-A expression in hippocampal neurons are blocked by the PKA inhibitor H8, implicating NGFI-A in the maternal care driven pathway mediated through PKA (Humblot et al., 1997; Mitchell et al., 1992). The non-coding exon 1 region of the expressed hippocampal GR gene includes a promoter sequence, exon 1_7, which contains a response element for NGFI-A (McCormick et al., 2000). The effect of 5-HT on NGFI-A expression, as well as the effect on GR levels, is mimicked by the 5-HT_7 receptor agonist 5-carboxytryptamine (5-CT). These effects are blocked by concurrent treatment with either ritanserin or ketanserin (5-$HT_{2/7}$ receptor agonist), but not by pindolol; a pharmacological profile that is consistent with the 5-HT_7 receptor (Humblot et al., 1997; Lovenberg et al., 1993a; Lovenberg et al., 1993b; Plassat et al., 1993). Splice variants of the GR mRNA transcripts containing the exon 1_7 sequence are found predominantly in the brain, and the expression of GR mRNAs transcripts containing the exon 1_7 sequence is increased in the hippocampus from offspring of High LG-ABN mothers or following manipulations that increase maternal LG-ABN behaviour. Importantly, the effects of maternal LG-ABN behaviour on hippocampal expression of NGFI-A are greatly reduced or completely eliminated in the offspring by pre-treatment with thyroid hormone synthesis inhibitors or 5-HT receptor antagonists. Furthermore, the effect on hippocampal cAMP levels is blocked, either by the 5-HT receptor

antagonist, ketanserin, or the thyroid hormone synthesis inhibitor propylthiouracil (PTU) (Meaney et al., 1987). Moreover, the thyroid hormone effect occurs only during the first weeks of life, a period that corresponds precisely to the known critical period for the effect of activity-dependent expression of GR in the hippocampus (Meaney and Aitken, 1985; Mellstrom et al., 1994). Accordingly, environmental enrichment early in life has been found to increase hippocampal expression of both NGFI-A and GR in the adult offspring (Mohammed et al., 1993; Sarrieau et al., 1988). Together, these findings are consistent with the notion that increased hippocampal NGFI-A expression drives the observed programming of hippocampal GR expression early in life.

9.1. *Experiment A*

In this experiment, we tested whether NGFI-A plays a dual role in regulating GR expression in response to maternal care. We hypothesize that in the adult, the transcriptional activator NGFI-A binding is inhibited by methylation of the 5′ CpG dinucleotide in its cognate response element, thus the differential state of methylation of this site in offspring of High and Low LG-ABN mothers results in differential GR promoter activation. NGFI-A transactivates the GR promoter by recruiting the histone acetyltransferase CBP, increasing histone acetylation a hallmark of gene activity. We also hypothesized that although NGFI-A binding is significantly reduced by DNA methylation, when NGFI-A is abundant binding of methylated DNA occurs resulting in targeting of CBP to methylated GR promoter region. The targeting of CBP results in histone acetylation and recruitment of demethylase machinery to the GR promoter. In this study, we tested all component of this hypothesis.

9.1.1. Animals and behaviour

The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Catherine's, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. For further methodological details, see section 4.1.

9.1.2. Semi-quantitative reverse transcription PCR (RT-PCR)

Same procedure as described before in section 4.13.

9.1.3. Chromatin immunoprecipitation (ChIP) assay

Same procedure as described before in section 4.10.

9.1.4. Sodium bisulfite mapping

Same procedure as described before in section 4.15.

9.1.5. Electrophoresis mobility shift assay (EMSA)

Same procedure as described before in section 4.11.

9.1.6. Hippocampal cell cultures and transient transfections

Same procedure as described before in section 4.16.

9.1.7. Western blotting analysis

Same procedure as described before in section 4.9.

9.1.8. HEK 293 cell cultures and transient transfections

Same procedure as described before in section 4.17.

9.1.9. Results

9.1.9.1. Early life maternal care effect on hippocampal exon 1_7 GR promoter expression

Differences in maternal LG-ABN behaviour occur between day 1 and 6 post-partum. We first tested the hypothesis that the differences in firing of exon 1_7 GR promoter are established during this time-period and remain permanent through life. RT-PCR analysis was performed using purified hippocampal mRNA from PND 6 (neonatal) and PND 90 (adult) offspring of High and Low LG-ABN dams (Figure 34a,b). There were significant Group effects for amount of GR mRNA expression in the neonatal [$t(1,14) = 3.4$, $^{*}P < 0.002$] and adult [$t(1,14) = 3.6$, $^{**}P < 0.001$] hippocampi, which indicated that levels of GR mRNA from the exon 1_7 promoter were greater in the neonatal and adult offspring of High LG-

ABN mothers in comparison to the neonatal and adult offspring of Low licking and grooming dams (Figure 34a,b). Global abundance of total protein in the hippocampus was not apparently affected, as indicated by the equal β-actin immunoreactivity (Figure 34a,b). These results suggest that the maternal care effects on hippocampal exon 1_7 GR promoter activities are established early in life and maintained through to adulthood.

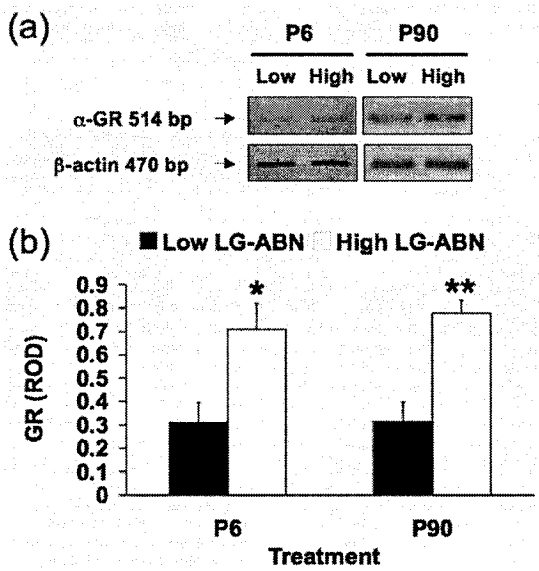

Figure 34: Maternal effect on hippocampal NGFI-A and GR mRNA expression in PND 6 (neonatal) and PND 90 (adult) animal (n = 10 animals/group). (**a**) Relative optical density (ROD; mean ± SEM) of hippocampal NGFI-A expression (*$P < 0.002$). (**b**) Mean ± SEM ROD of hippocampal GR expression (*$P < 0.001$).

9.1.9.2. Early life maternal effects on chromatin structure and NGFI-A binding

Previous data has established that maternal LG-ABN behaviour triggers a serotoninergic pathway activating PKA and stimulating cAMP, leading to activation of the transcription factor NGFI-A. Although the exon 1_7 GR promoter does not contain cAMP response element (CRE) sites, two NGFI-A response elements lie within the GR promoter sequence. NGFI-A has been previously shown to directly interact with the cAMP response element binding protein (CREB) binding protein (CBP) (Silverman et al., 1998). CBP is a HAT that activates chromatin by catalyzing histone acetylation. Histone acetylation at the lysine-9 (K9) residue of H3 and H4 histones is a well-established marker of active chromatin.

Acetylation of the histone tails neutralizes the positively charged histones, which disrupts histone binding to negatively charged DNA and thus promotes transcription factor binding. We hypothesize that NGFI-A interaction with the exon 1_7 GR promoter by day 6 post-partum precipitates a sequence of events resulting in DNA demethylation, which is a stable epigenetic modification that maintains the level of GR gene expression through life. We therefore tested the hypothesis that the maternal effect on DNA methylation resulted in (i) increased binding of NGFI-A protein to the promoter sequence; (ii) increased association between CBP and the exon 1_7 GR promoter region; and (iii) increased histone acetylation at the K9 residue of the H3 histone(s) associated with the promoter in the hippocampus of the 6 day pup. We performed a chromatin immunoprecipitation (ChIP) analysis of CBP association, histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter in the native chromatin environment *in vivo*. Intact hippocampi from 6-day-old offspring of High and Low LG-ABN mothers were crosslinked *in vivo* by paraformaldehyde perfusion. We then selectively immunoprecipitated protein-DNA complexes with either a CBP primary antibody, an acetylated H3-K9 histone primary antibody or an NGFI-A primary antibody. The protein-DNA complexes were uncrosslinked, and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence. There were significant Group effects for the association of CBP [$t(1,8) = 26.2$, $^{*}P < 0.05$], histone H3-K9 acetylation [$t(1,18) = 35.2$, $^{**}P < 0.001$] and NGFI-A [$t(1,8) = 42.1$, $^{***}P < 0.0001$] with the exon 1_7 GR promoter region (Figure 35). These results indicated significantly greater CBP association, histone H3-K9 acetylation and greater binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the neonatal offspring of High compared with Low LG-ABN mothers. Thus, maternal programming of the exon 1_7 GR promoter results in increased NGFI-A binding, CBP association and histone H3-K9 acetylation in the neonatal offspring.

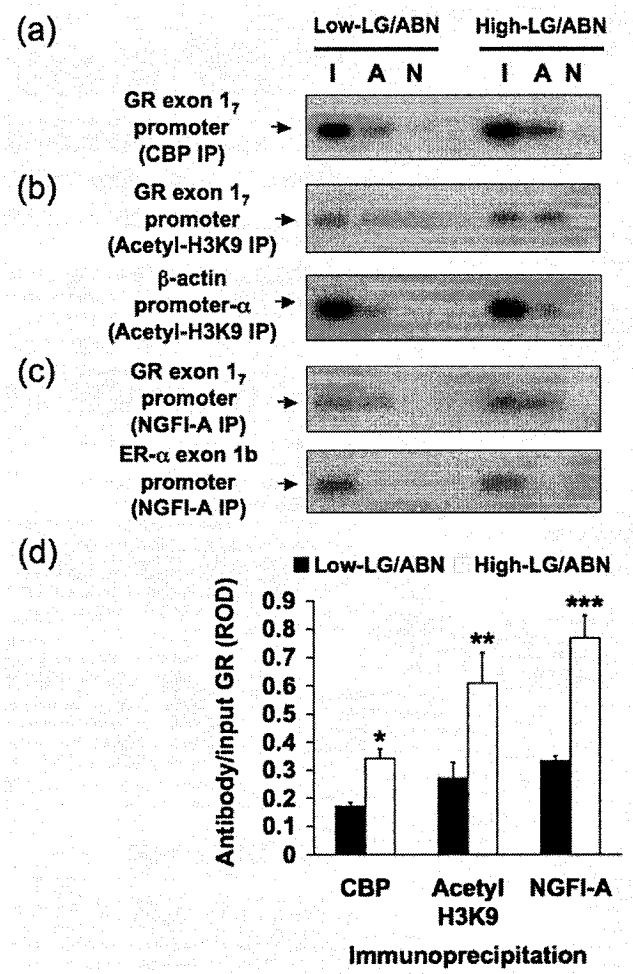

Figure 35: Chromatin immunoprecipitation analysis of the association between CBP, histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR sequence in hippocampal tissue of PND 6 High- and Low LG-ABN offspring (n = 4 animals/group). (**a**,**b**,**c**) Lanes were loaded with non-immunoprecipitated input (I); CBP, acetylated histone H3-K9 or NGFI-A primary antibody immunoprecipitated (A); or non-immune IgG antibody immunoprecipitated (N) hippocampal extracts. (**a**) Representative Southern blots of the amplified exon 1_7 region of the GR from CBP immunoprecipitated hippocampal tissue (194 bp band). (**b**) Representative Southern blots of the amplified exon 1_7 region from acetyl-histone H3-K9 immunoprecipitated hippocampal tissue (194 bp band) and β-actin (171 bp band) control. (**c**) Representative Southern blots of the amplified exon 1_7 region of the GR from NGFI-A immunoprecipitated hippocampal tissue (194 bp band). Exon 1b oestrogen receptor-α promoter region, which does not contain NGFI-A response elements (493 bp), amplified from the same NGFI-A immunoprecipitated hippocampal tissue was run as a control for specificity and showed no signal. (**d**) Relative optical density (ROD; mean ± SEM) of exon 1_7 sequence amplified from cAMP, acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue of PND 6 High- and Low LG-ABN offspring (*n* = 4 animals/group; **P < 0.05; **P < 0.001*; ****P < 0.0001*).

9.1.9.3. Hippocampal NGFI-A bound exon 1_7 GR promoter sodium bisulfite mapping

We have previously shown that maternal LG-ABN behaviour results in demethylation of the exon 1_7 GR promoter at day 6 post partum (Chapter 6). We first tested the hypothesis

that NGFI-A targets unmethylated exon 1_7 GR promoter sequences in day 6 hippocampi. Although we have previously shown that NGFI-A occupancy of the GR promoter is increased in adult offspring of High LG-ABN mothers where the gene is also less methylated, we did not determine whether the proteins selectively associates with the unmethylated copies of the gene. To establish the DNA methylation pattern of the exon 1_7 GR promoter sequence bound to the NGFI-A protein, sodium bisulfite mapping was performed using NGFIA immunoprecipitated hippocampal DNA from the neonatal offspring of High and Low LG-ABN mothers (Figure 36). Statistical analysis of the 5′ and 3′ CpG dinucleotides within the NGFI-A binding region of the GR promoter revealed a highly significant effect of Group [$F(1,8) = 53.7$, $P < 0.0001$], treatment [$F(1,8) = 65.8$, $P < 0.0001$] and Region [$F(1,8) = 112.2$, $P < 0.0001$], as well as a significant Group X Treatment interaction [$F(1,8) = 14.0$, $P < 0.0001$], Group X Region interaction [$F(1,8) = 42.8$, $P < 0.0001$] and treatment X Region interaction [$F(1,8) = 6.5$, $P = 0.04$]. *Post-hoc* analysis revealed that the 5′ and 3′ CpG dinucleotides within the NGFIA response element on the exon 1_7 GR promoter bound to NGFI-A were significantly demethylated in comparison to the non-immunoprecipitated 'Input' DNA in the offspring of High and Low LG-ABN mothers (*$P < 0.0001$ and **$P < 0.001$, respectively). It is interesting to note that although the exon 1_7 GR promoter is hypermethylated in the hippocampus of Low LG-ABN mothers in comparison with offspring of High LG-ABN mothers, the methylation status of the exon 1_7 promoter sequences that are bound to NGFI-A *in vivo* are equally unmethylated in both groups. Importantly, the cytosine residue within the 5′ CpG dinucleotide of the NGFI-A consensus sequence is always non-methylated when bound to NGFI-A protein regardless of maternal care. However, the maternal care treatment groups differ in the relative abundance of NGFI-A. Thus, the level of abundance of NGFI-A in the hippocampus determines the amount of exon 1_7 GR promoter sequences that are demethylated. This demonstrates that NGFI-A binding and demethylation exon 1_7 GR promoter in the day 6 hippocampi *in vivo* are tightly linked.

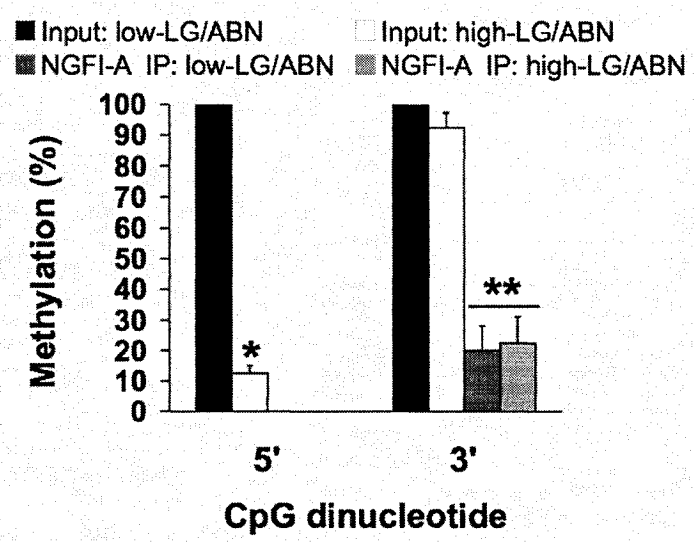

Figure 36: Mean ± SEM percentage methylation per cytosine for the 5' and 3' CpG dinucleotides within the NGFI-A binding region of the exon 1_7 GR promoter bound to NGFI-A protein PND 6 offspring of High and Low LG-ABN mothers (6-10 clones sequenced/animal; n = 4 animals/group; *$P < 0.0001$; **$P < 0.001$).

9.1.9.4. Site-specific methylation of the cytosine within the 5' CpG dinucleotide of the NGFI-A response element blocks transcription factor binding

This tight linkage between NGFI-A binding *in vivo* and the state of methylation of exon 1_7 GR promoter might be caused by two alternative but not mutually exclusive reasons. First, NGFI-A binding to its response element is inhibited by DNA methylation resulting in enrichment of unmethylated DNA bound to NGFI-A *in vivo*. DNA methylation affects gene expression either by attracting methylated DNA binding proteins to a densely methylated region of a gene (Nan et al., 1998) or by site-specific interference with the binding of a transcription factor to its response element (Comb and Goodman, 1990). Second, NGFI-A actively targets GR promoter for demethylation playing a causal role in its demethylation. Our previous data showing site-specific demethylation of the cytosine within the 5' CpG dinucleotide of the NGFI-A response element (Figure 17) in offspring of Low LG-ABN mothers is consistent with the hypothesis that methylation at this site interferes with the binding of NGFI-A protein to its response element. To address this question, we determined the *in vitro* binding of increasing concentrations (9 pmol, 18 pmol, 36 pmol) of purified recombinant NGFI-A protein (Milbrandt, 1987) to its response element under different

states of methylation using the electrophilic mobility shift assay (EMSA) technique with four ^{32}P-labelled synthetic oligonucleotide sequences (Figure 37a) bearing the NGFI-A binding site that was either i) non-methylated, ii) methylated in the 3′ CpG site, iii) methylated in the 5′ CpG site, iv) methylated in both sites or v) mutated at the two CpGs with an adenosine replacing the cytosines. NGFI-A formed a protein-DNA complex with the non-methylated oligonucleotide (Figure 37b, lanes 2-4), while the protein was unable to form a complex with either a fully methylated sequence or a sequence that was methylated at the 5′ CpG site (Figure 4b, lanes 10-12, 14-16). Partial activity was seen with the sequence methylated at the 3′ CpG site (Figure 37b, lanes 6-8). The specificity of the protein-DNA interaction is indicated by the fact that the recombinant protein fails to form a complex with the mutated NGFI-A response element, even at high protein concentrations (36 pmol) (Figure 37b lanes 18-20). This difference in binding was confirmed by competition experiments (Figure 37c). NGFI-A recombinant protein was incubated with a labelled, non-methylated oligonucleotide in the presence of an increasing concentrations of non-labelled oligonucleotides containing the NGFI-A consensus sequence that were either 3′ CpG methylated, 5′ CpG methylated, methylated at both sites, or mutated at the two CpG dinucleotides with an adenosine replacing the cytosines. As expected, the non-methylated oligonucleotide competes away the labelled oligonucleotide protein-DNA complex (Figure 37c, lane 7). The specificity of the protein-DNA interaction is indicated by the fact that the mutated oligonucleotide is unable to compete away the labelled oligonucleotide protein-DNA complex (Figure 37c lanes 17-19). Neither the oligonucleotide methylated in both the 3′ and 5′ CpG dinucleotides nor the 5′ CpG methylated oligonucleotide were able to compete (Figure 37c, lanes 11-16). Importantly, the 3′ CpG methylated oligonucleotide, which mimics the sequence observed in the offspring of High LG-ABN mothers (see Figure 36), exhibited substantial competition (Figure 37c, lanes 8-10). The results indicate that while methylation of the cytosine within the 5′ CpG dinucleotide reduced NGFI-A protein binding to the same extent as methylation in both CpG sites, methylation of the cytosine within

the 3′ CpG dinucleotide only partially reduced NGFI-A protein binding (Figure 37a,b). This data supports the hypothesis that methylation of the cytosine within the 5′ CpG dinucleotide within the NGFI-A response element of the exon 1_7 GR promoter region in the offspring of Low LG-ABN mothers inhibits NGFI-A protein binding, potentially explaining the reduced GR gene transcription in the offspring of the Low LG-ABN mothers.

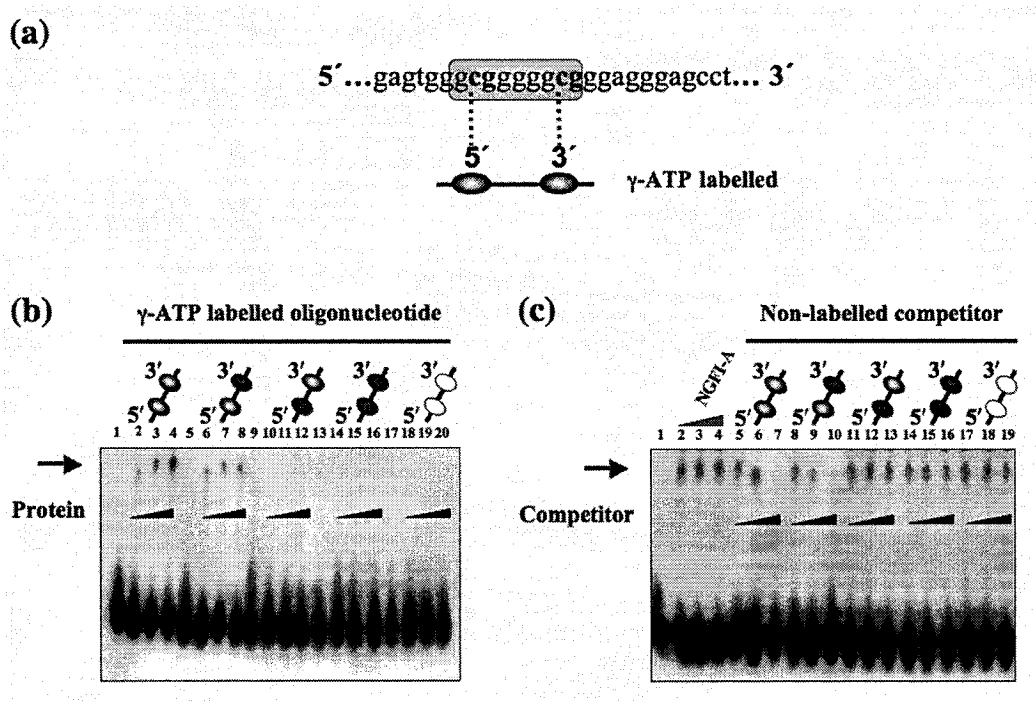

Figure 37: Purified NGFI-A EMSA. (**a**) The exon 1_7 GR promoter sequence with the NGFI-A response element (encircled). Beneath is a bead-on-string representation of a synthesized radiolabelled oligonucleotide probe, highlighting the two CpG dinucleotides [ovals represent the cytosines within the 5′ CpG and 3′ CpG dinucleotides] within the NGFI-A response element. (**b**) EMSA analysis of protein-DNA complex formation between recombinant purified NGFI-A protein and radiolabelled oligonucleotide (shown in Figure 37a) bearing the NGFI-A response element containing differentially methylated cytosines within the 5′ CpG and 3′ CpG dinucleotides. Non-methylated cytosines are represented by grey ovals, methylated cytosines are shown as black ovals and white ovals are mutated CpG dinucleotides with an adenosine replacing the cytosine. The presence of increasing amounts of purified NGFI-A protein (9 pmol, 18 pmol, or 36 pmol) is indicated by the black triangle. The black arrow indicates the shift in labelled oligonucleotide mobility. Lane 1: free oligonucleotide non-methylated at either dinucleotide. Lanes 2-4: non-methylated oligonucleotide with an increasing amount of NGFI-A. Lane 5: free oligonucleotide methylated at the 3′ CpG dinucleotide. Lanes 6-8: oligonucleotide methylated at the 3′ CpG dinucleotide with an increasing amount of NGFI-A. Lane 9: free oligonucleotide methylated at the 5′ CpG dinucleotide. Lanes 10-12: oligonucleotide methylated at the 5′ CpG dinucleotide with an increasing amount of NGFI-A. Lane 13: free oligonucleotide methylated at both 5′ and 3′ CpG dinucleotides. Lanes 14-16: oligonucleotide methylated at both 5′ and 3′ CpG dinucleotides with an increasing amount of NGFI-A. Lane 17: free non-methylated oligonucleotide mutated with an adenosine replacing the cytosine in both the 5′ and 3′ CpG dinucleotides. Lanes 18-20: mutated non-methylated oligonucleotide with increasing amount of NGFI-A. Note that methylation of the cytosine within the 3′ CpG dinucleotide

reduced binding at the higher levels of NGFI-A protein, while methylation of the cytosine within the 5′ CpG dinucleotide completely eliminated protein binding to the NGFI-A response element. (**c**) EMSA analysis of competition of protein-DNA complex formation between NGFI-A protein and a radiolabelled oligonucleotide probe containing the NGFI-A response element (shown in Figure 37a) by an excess of non-labelled oligonucleotides containing differentially methylated cytosines within the 5′ and 3′ CpG dinucleotides of the NGFI-A response element. Non-methylated cytosines are represented by grey ovals, methylated cytosines are shown as black ovals and white ovals are mutated CpG dinucleotides with an adenosine replacing the cytosine. The presence of increasing amounts of purified NGFI-A protein (9 pmol, 18 pmol, or 36 pmol) is indicated by the grey triangle. The presence of increasing amount of non-labelled oligonucleotide competitor (1:100 fold, 1:500 fold, or 1:1000 fold) is indicated by the black triangle. The black arrow indicates the shift in labelled oligonucleotide mobility. Lane 1: free labelled non-methylated oligonucleotide. Lanes 2-4: labelled non-methylated oligonucleotide with increasing amount of NGFI-A. Lanes 5-7: labelled non-methylated oligonucleotide with increasing amount of non-labelled non-methylated oligonucleotide competitor. Lanes 8-10: labelled non-methylated oligonucleotide with increasing amount of non-labelled oligonucleotide competitor methylated at the 3′ CpG dinucleotide. Lanes 11-13: labelled non-methylated oligonucleotide with increasing amount of non-labelled oligonucleotide competitor methylated at the 5′ CpG dinucleotide. Lanes 14-16: labelled non-methylated oligonucleotide with increasing amount of non-labelled oligonucleotide competitor methylated at both 5' and 3' CpG dinucleotides. Lanes 17-19: labelled non-methylated oligonucleotide with increasing amount of non-labelled oligonucleotide competitor non-methylated but mutated with an adenosine replacing the cytosine in both the 5′ and 3′ CpG sites. Note that only the oligonucleotides with a non-methylated cytosine at the 5′ CpG dinucleotide of the NGFI-A response element showed effective competition. Methylation of the cytosine within the 5' CpG dinucleotide completely eliminated the ability of the non-labelled oligonucleotides to compete for NGFI-A protein binding to the radio-labelled, non-methylated oligonucleotide sequence containing the NGFI-A response element.

9.1.9.5. Effects of 5-HT, cAMP and NGFI-A on hippocampal GR promoter programming

While our *in vivo* studies described above are consistent with the hypothesis that increased activation of 5-HT receptors and the resulting activation of cAMP levels result in increased NGFI-A binding to exon 1_7 sequence and that the increased GR promoter occupancy with NGFI-A is linked to its unmethylated state they did not define a causal relationship. We therefore used a hippocampal cell culture system to first examine whether NGFI-A binding to the exon 1_7 GR promoter is mediated by 5-HT and cAMP levels and secondly to determine whether NGFI-A plays a causal role in the epigenetic programming of the GR promoter. Hippocampal cell cultures from ED 19-20 Long-Evans rat foetuses were prepared as previously described (Laplante et al., 2002). Two days after seeding, uridine (20 mM) and 5-fluorodeoxyuridine (20 mM) were added to the medium to prevent proliferation of glial cells. The cultures then received a single treatment of 5H-T (100 nM) or 8-bromo cAMP (10 mM). Four days later, the cells were harvested to determine levels of GR pro-

moter methylation (Figure 38a). Statistical analysis of the data from the 5′ and 3′ CpG dinucleotides revealed a significant effect of Treatment [$F(1,14) = 12.8$, $P < 0.001$] and Region [$F(1,14) = 45.3$, $P < 0.001$], as well as a significant Treatment X Region interaction [$F(1,14) = 5.1$, $P = 0.04$]. *Post-hoc* analysis revealed that, though no significant differences were found for the 3′ CpG dinucleotide, the 5′ CpG dinucleotide was significantly demethylated in the 5-HT and cAMP-treated cultures compared to the non-treated cultures (*$P < 0.001$; Figure 38a). These results suggest that 5-HT causes demethylation of the 5′ CpG dinucleotide within the NGFI-A response element of the GR promoter. We next used NGFI-A antisense oligonucleotides to determine whether NGFI-A targeted the downstream effects of 5-HT on exon 1_7 GR promoter methylation and gene expression. The cultures received a single treatment of 5H-T (100 nM) followed by NGFI-A antisense (1 μM) oligonucleotide. Cultures were treated with 5H-T (100 nM) followed by scrambled missense (1 μM) to control for non-specific oligonucleotide effects. Four days later, the cells were harvested to determine levels of GR promoter methylation and gene expression (Figure 38b-d). Statistical analysis of the data from the 5′ and 3′ CpG dinucleotides revealed a significant effect of Treatment [$F(1,14) = 27.8$, $P < 0.0001$] and Region [$F(1,14) = 31.93$, $P < 0.0001$], as well as a significant Treatment X Region interaction [$F(1,14) = 1.98$, $P = 0.003$]. *Post-hoc* analysis revealed that, though no significant differences were found for the 3′ CpG dinucleotide, the 5′ CpG dinucleotide was significantly demethylated in the 5-HT/scrambled missense oligonucleotide-treated cultures (*$P < 0.001$; Figure 38b) compared to the non-treated and 5-HT/NGFI-A antisense oligonucleotide-treated cultures (5-HT/scrambled missense Vs. non-treated; 5-HT/scrambled missense Vs. 5-HT/NGFI-A antisense). Thus, NGFI-A antisense oligonucleotide treatment eliminated the effects of 5-HT treatment on methylation of the 5′ CpG dinucleotide within the NGFI-A binding region of the GR promoter ($P > 0.05$). Subsequent western blot analysis showed that NGFI-A antisense oligonucleotide treatment also blocked the effect of 5-HT treatment on GR protein expression [$F(1,16) = 2.3$, *$P < 0.001$; Figure 38c-d].

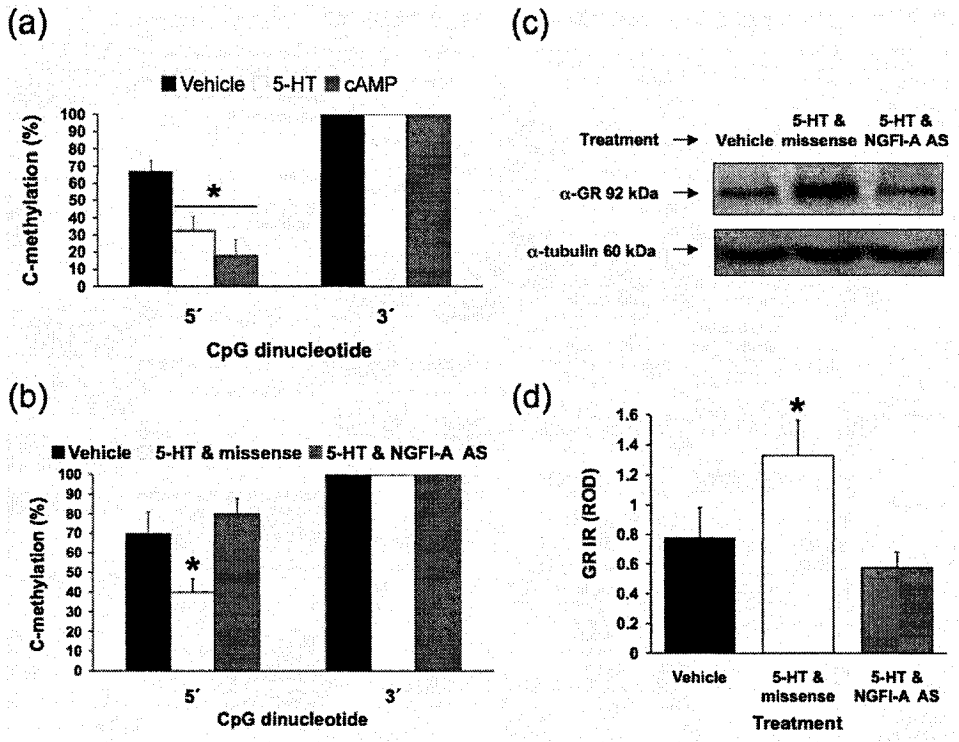

Figure 38: Effects of 5HT, cAMP and NGFI-A on hippocampal GR promoter methylation and gene expression. (**a**) Mean ± SEM percentage methylation per cytosine for the 5′ and 3′ CpG dinucleotides within the NGFI-A binding region of the GR promoter from 5-HT and cAMP-treated hippocampal cell culture (6-10 clones sequenced/sample; n = 4 samples/treatment; *$P < 0.001$). (**b**) Mean ± SEM percentage methylation per cytosine for the 5′ and 3′ CpG dinucleotides within the NGFI-A binding region of the GR promoter from 5-HT/NGFI-A missense and 5-HT/NGFI-A antisense-treated hippocampal cell culture (6–10 clones sequenced/sample; n = 4 samples/treatment; *$P < 0.001$). (**c**,**d**) Western blot analysis of GR expression from non-treated, 5-HT/NGFI-A missense and 5-HT/NGFI-A antisense-treated hippocampal cell culture (6–10 clones sequenced/sample; n = 4 samples/treatment; *$P < 0.001$).

Hippocampal cell cultures treated with either 5-HT or 8-bromo-cAMP, a stable cAMP analogue, show increased GR expression and hypomethylation of the 5′ CpG dinucleotide of the NGFI-A response element within the exon 1_7 GR promoter, with no effect at the 3′ CpG dinucleotide in the absence of cell replication. Cultures maintained under control conditions show heavy methylation of both the 5′ and 3′ CpG dinucleotide of the NGFI-A response element. Bromodeoxyuridine (BrdU) labelling, which marks newly generated cells, reveals little or no cell replication in the cultures at the time of 5-HT treatment; indeed, the cultures were treated with mitotic inhibitors to prevent glial proliferation. In

summary, our studies demonstrate that NGFI-A plays a causal role in the state of activity and programming of the exon 1_7 GR promoter.

9.1.9.6. Effects of NGFI-A activity on exon 1_7 GR promoter expression

Though the hippocampal culture studies established a causal relationship between NGFI-A expression and the epigenetic status of the exon 1_7 GR promoter, they did not address three important questions. First, does GR promoter activation by NGFI-A involve direct interaction with the exon 1_7 sequence? Second, can high levels of NGFI-A demethylate the exon 1_7 sequence and activate the GR promoter? Third, does NGFI-A induce demethylation of the exon 1_7 sequence through an active or passive process? To address these questions we transiently transfected the exon 1_7 GR promoter: luciferase vector in either the absence or presence of NGFI-A overexpression into HEK 293 cells. Furthermore, to test whether the exon 1_7 GR promoter activity requires direct contact between NGFI-A and its response element, we performed site directed mutagenesis of the two CpG dinucleotides within the NGFI-A response element. Seventy-two hours following transfection, the cells were harvested to determine levels of luciferase gene expression (Figure 39a). Statistical analysis revealed a significant Group X Treatment interaction [$F(1,16) = 1.98,\ P = 0.004$] for exon 1_7 GR promoter driven luciferase expression. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased luciferase gene expression in cells cotransfected with non-mutated ($*P < 0.001$) or 5′ CpG dinucleotide mutated ($**P < 0.03$) exon 1_7 GR promoter (Figure 39a). There was no significant effect of NGFI-A overexpression on luciferase gene induction in cells cotransfected with 3′ CpG dinucleotide mutated exon 1_7 GR promoter ($P > 0.05$). Thus, the 3′ CpG dinucleotide within the NGFI-A response element on the exon 1_7 sequence appears to be crucial for induction of the GR promoter by NGFI-A. Using these same samples, we then selectively immunoprecipitated protein-DNA complexes with either a CBP primary antibody, an acetylated H3-K9 histone primary antibody or an NGFI-A primary antibody. The protein-DNA complexes were uncrosslinked,

and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence (Figure 39b-d). Statistical analysis revealed a significant Treatment x Group interaction effect for the association of CBP [$F(1,16) = 4.93$, $P < 0.05$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased CBP association with the exon 1_7 region in cells cotransfected with non-mutated ($*P < 0.001$) or 5′ CpG dinucleotide mutated ($**P < 0.05$) GR promoter sequences (Figure 39b). Interestingly, the 3′ CpG dinucleotide mutation abolished the interaction of CBP with exon 1_7 GR promoter illustrating that physical interaction of NGFI-A with the 3′ CpG dinucleotide is a prerequisite for CBP interaction with the promoter and the consequential histone acetylation. Similarly, there was significant Treatment x Group interaction effect for the association of histone H3-K9 acetylation [$F(1,16) = 4.93$, $P < 0.05$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased histone H3-K9 acetylation of the exon 1_7 region in cells cotransfected with non-mutated ($*P < 0.001$) or 5′ CpG dinucleotide mutated ($**P < 0.05$) GR promoter sequences (Figure 39c). There was also significant Treatment x Group interaction effect for the association of NGFI-A [$F(1,16) = 8.97$, $P = 0.01$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased histone H3-K9 acetylation of the exon 1_7 region in cells cotransfected with non-mutated ($*P < 0.01$) or 5′ CpG dinucleotide mutated ($**P < 0.05$) exon 1_7 GR promoter sequences (Figure 39d). Together, these studies revealed that NGFI-A overexpression in cells cotransfected with the 3′ CpG dinucleotide mutated exon 1_7 sequence showed no significant ($P > 0.05$) effect on CBP, histone H3-K9 acetylation and NGFI-A association with the GR promoter. Thus, the 3′ CpG dinucleotide appears to be crucial for the association of CBP association, histone H3-K9 acetylation and NGFI-A binding with the exon 1_7 region and induction of GR promoter activation.

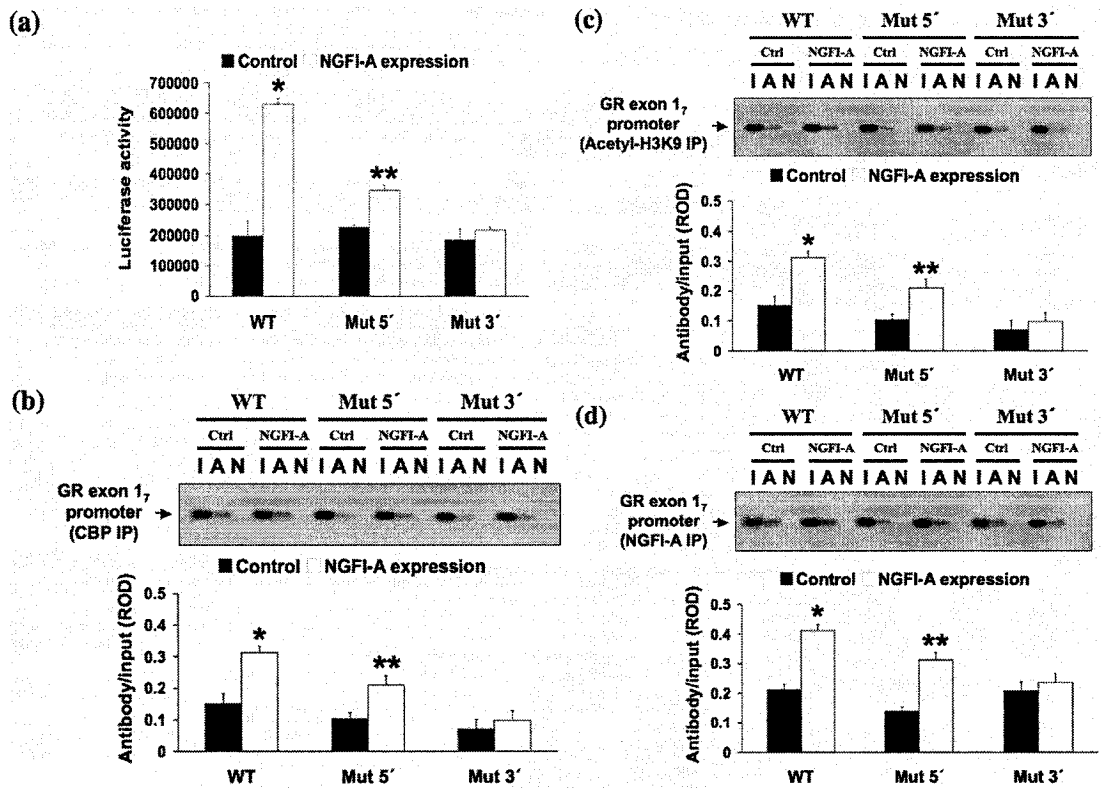

Figure 39: Effects of mutation of the NGFI-A response element on exon 1_7 GR promoter function. (**a-d**) Luciferase expression, CBP association, histone H3-K9 acetylation and NGFI-A binding of the exon 1_7 GR promoter-luciferase reporter plasmid containing either a wild type (WT) non-mutated, 5′ CpG mutated or 3′ CpG mutated NGFI-A response element, co-transfected without or with an NGFI-A expression plasmid (n = 4 samples/treatment). (**a**) Mean ± SEM luciferase expression (*$*P < 0.001$*; *$**P < 0.03$*). (**b**) Mean ± SEM relative optical density (ROD) of exon 1_7 sequence amplified from CBP immunoprecipitated cell extract (*$*P < 0.001$*; *$**P < 0.05$*). (**c**) Mean ± SEM ROD of exon 1_7 sequence amplified from histone H3-K9 acetylation immunoprecipitated cell extract (*$*P < 0.01$*; *$**P < 0.05$*). (**d**) Mean ± SEM ROD of exon 1_7 sequence amplified from NGFI-A immunoprecipitated cell extract (*$*P < 0.01$*; *$**P < 0.5$*).

9.1.9.7. Effects of ectopic NGFI-A expression on methylated GR promoter

We have previously demonstrated (Cervoni and Szyf, 2001) that a methylated plasmid lacking an origin of replication does not replicate in human embryonic kidney (HEK) 293 cells. Therefore, any change in methylation of a fully methylated plasmid during a transient transfection experiment must involve replication-independent active demethylation. To test whether exon 1_7 GR promoter activation is inhibited by methylation and whether NGFI-A activates and demethylates the methylated GR exon 1_7 promoter, we *in vitro* methylated the GR exon 1_7 promoter and the reporter plasmids bearing site specific mutations

in either the 5′ or 3′ CpG dinucleotides within the NGFI-A response element and transfected them into HEK 293 cells in either the absence or presence of ectopic expression of NGFI-A. These site-specific mutations substituted the cytosines in the CpG dinucleotide with an adenine therefore prohibiting methylation in these sites. Seventy-two hours following transfection, the cells were harvested to determine levels of luciferase gene expression from the exon 1_7 GR promoter in living cells (Figure 40). Statistical analysis revealed a significant Group X Treatment interaction [$F(1,16) = 4.86$, $P = 0.005$] for GR exon 1_7 promoter driven luciferase expression. Our data reveals that global methylation of the reporter plasmid suppresses the expression. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased luciferase gene expression in cells cotransfected with methylated and non-mutated ($*P < 0.001$) or methylated and 5′ CpG dinucleotide mutated ($**P < 0.0002$) exon 1_7 GR promoter (Figure 40). There was no significant effect of NGFI-A overexpression on luciferase gene induction in cells cotransfected with 3′ CpG dinucleotide mutated and methylated exon 1_7 GR promoter ($P > 0.05$).

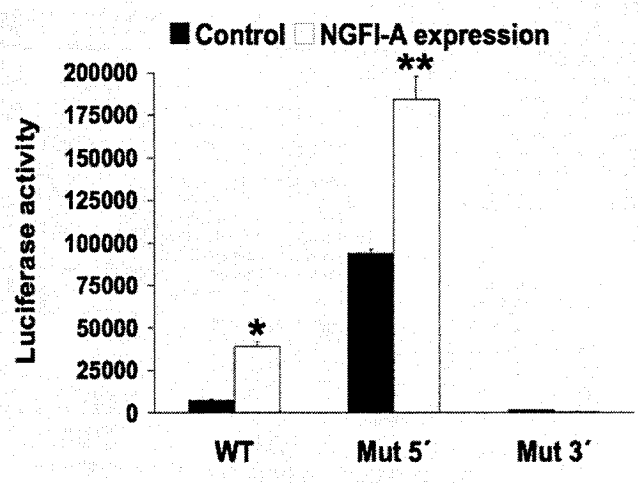

Figure 40: Effects of methylation of the NGFI-A response element on exon 1_7 GR promoter function. Mean ± SEM of luciferase expression ($*P < 0.001$; $**P < 0.0002$) from the methylated exon 1_7 GR promoter-luciferase reporter plasmid containing either a wild type (WT) non-mutated, 5′ CpG mutated or 3′ CpG mutated NGFI-A response element, co-transfected without or with an NGFI-A expression plasmid (n = 4 samples/treatment).

Taken together with the *in vitro* binding assay (Figure 37b,c), these results indicate that although methylation blocks NGFI-A binding (Figure 37b,c) and reduces expression of exon 1_7 GR promoter (Figure 40), high ectopic expression of NGFI-A can reverse the methylation mediated inhibition of transcription and activates the methylated exon 1_7 GR promoter (Figure 40). This is consistent with our working hypothesis that NGFI-A is involved in the initial activation and reprogramming of methylated exon 1_7 GR promoter on PND 6. Interestingly, mutation of the 5′ CpG dinucleotide within the NGFI-A response element to an ApG dinucleotide, essentially rendering the site non-methylated, partially relieved the inhibition by DNA methylation and enabled NGFI-A induction of the exon 1_7 GR promoter (Figure 40). This suggests that the 5′ CpG dinucleotide within the NGFI-A response element functions as a methylation sensor and its methylation status is critical for silencing of the gene by methylation, as the presence of a cytosine nucleotide within the 5′ CpG dinucleotide appeared to be unimportant for activation by NGFI-A (Figure 40). On the other hand, promoter sequences bearing the mutation of the 3′ CpG dinucleotide to ApG dinucleotide, essentially rendering the site non-methylated, are silent and non-inducible with NGFI-A. This suggests that the nucleotide sequence, and not the methylation status, of the 3′ CpG dinucleotide is critical for exon 1_7 GR promoter activation by NGFI-A. Together, these findings suggest that the methylation status of the 5′ CpG dinucleotide and the presence of the 3′ CpG dinucleotide within the NGFI-A response element are critical for NGFI-A activation of the methylated exon 1_7 GR promoter .

Thus far, our hypothesis has been that CBP acetyltransferase is recruited to the exon 1_7 GR promoter following NGFI-A interaction with its cognate response element. To test the hypothesis we selectively immunoprecipitated protein-DNA complexes from the previously described methylated exon 1_7 GR promoter-luciferase reporter cotransfection with either a CBP primary antibody, an acetylated H3-K9 histone primary antibody or an NGFI-A primary antibody. The protein-DNA complexes were uncrosslinked, and the precipitated

genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence (Figure 41a-c). Statistical analysis revealed a significant Treatment x Group interaction effect for the association of CBP [$F(1,16) = 5.63$, $P < 0.05$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased CBP association with the exon 1_7 region in cells cotransfected with methylated and non-mutated ($*P < 0.05$) or methylated and 5′ CpG dinucleotide mutated ($**P < 0.01$) GR promoter sequences (Figure 41a). Similarly, there was significant Treatment x Group interaction effect for the association of histone H3-K9 acetylation [$F(1,16) = 4.93$, $P < 0.05$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased histone H3-K9 acetylation of the exon 1_7 region in cells cotransfected with methylated and non-mutated ($*P < 0.05$) or methylated and 5′ CpG dinucleotide mutated ($**P < 0.001$) GR promoter sequences (Figure 41b). There was also significant Treatment x Group interaction effect for the association of NGFI-A [$F(1,16) = 8.97$, $P = 0.01$] with the exon 1_7 GR promoter. *Post-hoc* analysis revealed that NGFI-A overexpression significantly increased histone H3-K9 acetylation of the exon 1_7 region in cells cotransfected with methylated and non-mutated ($*P < 0.05$) or methylated and 5′ CpG dinucleotide mutated ($**P < 0.0001$) exon 1_7 GR promoter sequences (Figure 41c). Thus, mutation of the 3′ CpG dinucleotide appears to be crucial for the association of NGFI-A, CBP, histone H3-K9 acetylation and with the methylated exon 1_7 GR promoter sequence.

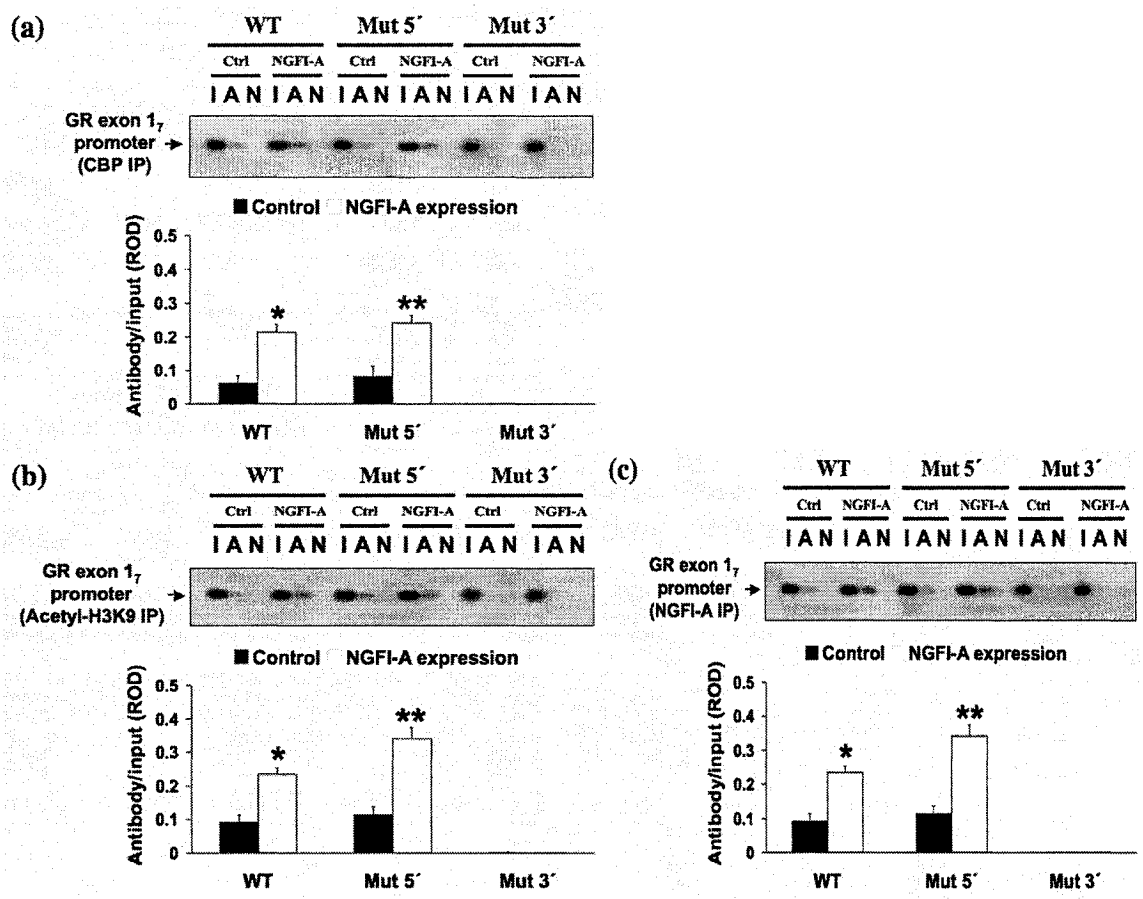

Figure 41: Effects of methylation of the NGFI-A response element on exon 1_7 GR promoter function. (**a-c**) CBP association, histone H3-K9 acetylation and NGFI-A binding to the methylated exon 1_7 GR promoter-luciferase reporter plasmid containing either a wild type (WT) non-mutated, 5′ CpG mutated or 3′ CpG mutated NGFI-A response element, co-transfected without or with an NGFI-A expression plasmid (n = 4 samples/treatment). (**a**) Mean ± SEM relative optical density (ROD) of exon 1_7 sequence amplified from CBP immunoprecipitated cell extract (*$P < 0.05$; **$P < 0.01$). (**b**) Mean ± SEM ROD of exon 1_7 sequence amplified from histone H3-K9 acetylation immunoprecipitated cell extract (*$P < 0.05$; **$P < 0.001$). (**c**) Mean ± SEM ROD of exon 1_7 sequence amplified from NGFI-A immunoprecipitated cell extract (*$P < 0.05$; **$P < 0.0001$).

Our data shows that under conditions where ectopic NGFI-A is expressed in the cell it is able to interact with the methylated exon 1_7 GR promoter, recruit CBP and bring about histone acetylation. When the 3′ CpG dinucleotide is mutated, this interaction does not take place and CBP is not recruited. This data is consistent with the hypothesis that interaction between NGFI-A and the methylated exon 1_7 GR promoter during early postnatal life epigenetically programmes the GR promoter.

9.1.9.8. Over-expressed NGFI-A causes demethylation of methylated exon 1_7 GR promoter as determined by sodium bisulfite mapping

We tested the hypothesis that direct interaction of NGFI-A with the methylated exon 1_7 sequence results in GR promoter demethylation. Loss of methylation could be caused by either passive demethylation whereby nascent DNA molecule is synthesized in the absence of DNA methyltransferase during replication resulting in an unmethylated strand of DNA or by an active removal of methyl groups from DNA in the absence of cell division. Since the exon 1_7 GR promoter-luciferase reporter is a plasmid which does not bear an origin of replication and does not replicate in HEK 293 cells during the period of transient transfection (Cervoni and Szyf, 2001), this system could be used to determine whether NGFI-A brings about active demethylation of the exon 1_7 GR promoter. We first mapped the state of methylation of exon 1_7 GR promoter sequence after seventy-two hours of transient transfection in either the presence or absence of ectopic expression of NGFI-A. Our data shows that ectopic expression of NGFI-A resulted in 50 % demethylation of the co-transfected exon 1_7 GR promoter. To determine whether this demethylation requires the interaction of NGFI-A with the exon 1_7 GR promoter we tested whether ectopic NGFI-A would cause demethylation of the cotransfected 3′ CpG dinucleotide GR promoter-luciferase mutant that does not bind NGFI-A. Our data shows that a mutation that abolishes the binding of NGFI-A also prevents demethylation.

To further establish that direct interaction between NGFI-A and exon 1_7 GR exon promoter is required for demethylation we tested whether NGFI-A bound exon 1_7 GR promoter is unmethylated as is expected if interaction between NGFI-A and methylated exon 1_7 GR promoter causes demethylation. Sodium bisulfite mapping was performed using NGFI-A immunoprecipitated DNA from the previously described methylated exon 1_7 GR promoter-luciferase reporter cotransfection (Figure 42a,b). Statistical analysis of the data from the 5′ and 3′ CPG dinucleotides revealed a highly significant effect of Group [$F(1,32) = 42.5$, $P < 0.0001$], Treatment [$F(1,32) = 58.6$, $P < 0.0001$] and Region [$F(1,32) = 110.2$, $P < 0.0001$], as well as a significant Group X Treatment interaction [$F(1,32) = 14.0$, $P <$

0.0001], Group X Region interaction [$F(1,32) = 36.7$, $P < 0.0001$] and Treatment X Region interaction [$F(1,32) = 6.2$, $P = 0.04$]. *Post-hoc* analysis revealed that NGFI-A overexpression significantly ($^*P < 0.001$) increased DNA demethylation in cells cotransfected with methylated and non-mutated exon 1_7 GR promoter (Figure 42a). There was no significant effect ($P > 0.05$) of NGFI-A overexpression on DNA demethylation in cells cotransfected with methylated and 3′ CpG dinucleotide mutated exon 1_7 GR promoter (Figure 42b). Thus, NGFI-A overexpression ultimately leads to demethylation of the 5′ CpG dinucleotide within the NGFI-A response element of the exon 1_7 GR promoter construct. The 3′ CpG dinucleotide within the NGFI-A response element of the methylated exon 1_7 sequence appears to be crucial for demethylation of the GR promoter in the presence of NGFI-A. Indeed, NGFI-A bound exon 1_7 GR promoter sequences are almost completely demethylated in NGFI-A cotransfectants (Figure 42a,b). The enrichment of demethylated molecules in the NGFI-A bound fraction supports the hypothesis that interaction of NGFI-A with methylated exon 1_7 GR promoter results in demethylation, the remaining methylated molecules in the input must represent a fraction that do not interact with NGFI-A. In summary, our data strongly supports the hypothesis that direct interaction between NGFI-A and methylated exon 1_7 GR promoter targets active demethylation to this sequence.

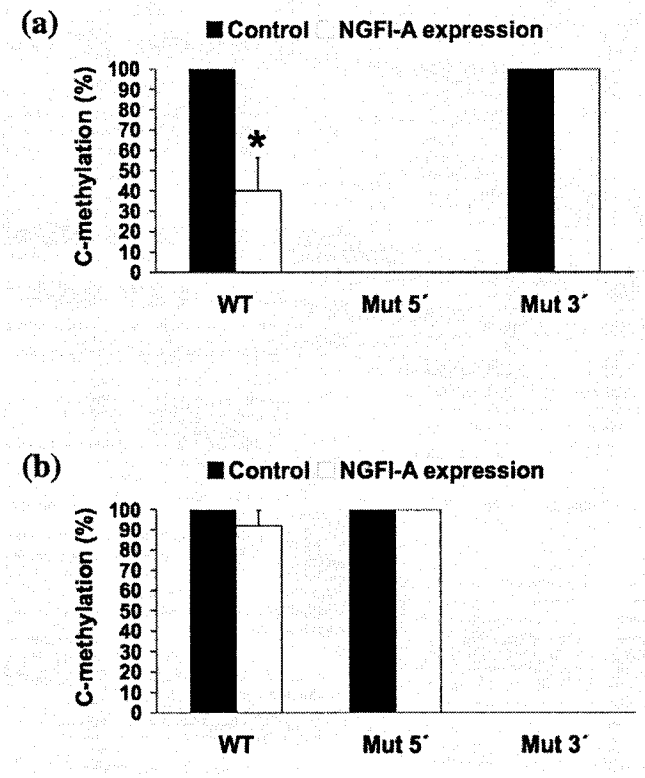

Figure 42: Effects of mutations at the NGFI-A response element on active demethylation of GR promoter. (**a,b**) Percentage methylation per cytosine for the two CpG dinucleotides within the NGFI-A response element of the exon 1_7 GR promoter bound to NGFI-A immunoprecipitated cell extract from the cotransfection shown in Figure 8c (6-10 clones sequenced/sample; n = 4 samples/treatment). (**a**) Mean ± SEM percentage methylation per cytosine for the 5′ CpG dinucleotide (*$P < 0.001$). (**b**) Mean ± SEM percentage methylation per cytosine for the 3′ CpG dinucleotide.

9.2. *Discussion*

Maternal programming by NGFI-A mediated DNA methylation

We have previously shown that increased maternal LG-ABN is associated with increased GR mRNA expression from the exon 1_7 promoter in the hippocampus of the neonate, which is sustained into adulthood (Chapter 6). We proposed that this differential expression is epigenetically programmed by maternal care. In accordance with this hypothesis, we have previously shown that adult offspring of High LG-ABN mothers were found to have higher occupancy of the exon 1_7 GR promoter with the transcription factor NGFI-A, elevated histone acetylation and demethylation of the 5′ CpG dinucleotide within the NGFI-A response element. However, it remained unclear how these events are causally-related

and how maternal care might possibly result in epigenetic reprogramming. Demethylation takes place between PND 1 and 6 over the period of intense maternal LG-ABN behaviour. NGFIA expression peaks in response to maternal care during this time period, while the level of its expression later in life do not differ between the High and Low LG-ABN offspring. The study presented here tested whether the hypothesis the transcription factor NGFI-A is plays a crucial role in both the epigenetic reprogramming during PND 1 to 6 and in maintaining the highly active state of exon 1_7 GR promoter in a methylation dependent manner through to adult life. This hypothesis implies the following assumptions. The first set of assumptions relates to the role of NGFIA in differentially regulating hippocampal GR expression in the adult animal. First, it is proposed that interaction of NGFI-A with its cognate response element is inhibited by DNA methylation, explaining the increased NGFIA occupancy on the GR promoter in adult offspring of High LG-ABN mothers whose GR promoter is unmethylated. This, notwithstanding the absence of divergence in overall levels of NGFI-A between maternal care groups in the adult hippocampus. The second supposition is that NGFI-A recruits HATs to the GR promoter thus explaining the increased acetylation of the exon 1_7 GR promoter in offspring of High LG-ABN mothers. Third, increased acetylation results in increased GR expression. We used a combination of *in vivo,* primary hippocampal culture cell culture and *in vitro* studies to test this hypothesis. We show using an *in vitro* EMSA assay that methylation of the 5′ CpG dinucleotide within the NGFI-A response element blocks binding of NGFI-A to the exon 1_7 GR promoter sequence *in vitro*. *In vivo* the exon 1_7 GR promoter, which is bound to NGFI-A, is unmethylated in both High and Low LG-ABN offspring. Although the offspring of Low LG-ABN mothers express low levels of hippocampal GR and most of the exon 1_7 GR promoter sequences in their hippocampi remain methylated, the small fractions of promoter sequences that are still bound to NGFI-A in these animals remain unmethylated. This demonstrates that *in vivo* NGFI-A binds only to unmethylated DNA.

Ectopic expression of NGFI-A in HEK 293 cells induces cotransfected exon 1_7 GR promoter-luciferase vector expression and this induction is abolished when the 3′ CpG dinucleotide within the NGFI-A response element of exon 1_7 GR promoter is mutated, demonstrating that binding of NGFI-A to the exon 1_7 sequence is required for GR promoter activation. *In vivo* the level of occupancy of the exon 1_7 GR promoter with CBP is correlated with the level of NGFI-A binding and in cell culture. CBP binding to the exon 1_7 GR promoter requires an intact NGFI-A response element, demonstrating that NGFI-A binding recruits CBP. Mutation of the 3′ CpG dinucleotide inhibits CBP association. Previous data have shown that CBP interacts with NGFI-A. CBP is a HAT and is probably responsible for the state of acetylation of GR. The interaction of NGFI-A with GR in cultured cells as revealed by ChIP assays as well as the activity of the reporter exon 1_7 GR promoter-luciferase is inhibited by DNA methylation. In accordance with the *in vitro* data we show that mutation of the 5′ CpG dinucleotide to ApG dinucleotide, which abolishes its ability to be methylated, increases the basal activity of methylated exon 1_7 GR promoter-luciferase vector and enables partial activation with NGFI-A, supporting the hypothesis that methylation of this site plays a critical role in suppressing NGFI-A interaction and the activity of the GR promoter.

CBP is known to serve as a scaffold protein for complexes of transcription factors and is involved inactivating numerous promoters. It has been previously shown to activate promoters, which are also targeted by NGFI-A. Combined, this data provides a mechanism for regulation of GR by the state of DNA methylation and illustrates how a differential state of methylation of the 5′ CpG dinucleotide within the NGFI-A response element in the exon 1_7 GR promoter established early after birth, which defines the state of activity later in life. Methylation of the 5′ CpG dinucleotide within the NGFI-A response element blocks its interaction with exon 1_7 GR promoter. Since interaction of NGFI-A with unmethylated exon 1_7 GR promoter recruits CBP to the promoter and increases histone acetylation as well

as promotes transcriptional activation, lack of NGFI-A binding inhibits GR expression. In offspring of High maternal LG-ABN in a high fraction of the hippocampal cells exon 1_7 GR promoters are unmethylated and bound to NGFI-A. In contrast, in Low LG-ABN offspring most of the promoters are methylated and unbound to NGFI-A resulting in low level of firing of the exon 1_7 GR promoter. While these data explain how the differential methylation of GR results in differential activity of the exon 1_7 GR promoter, we are left with the question of how differences in the pattern of DNA methylation are established early in life.

We previously observed that NGFI-A is elevated early in life in response to the 5-HT signalling pathway induced by High LG-ABN. This prompted us to test the hypothesis that high levels of NGFI-A induced by maternal care target the exon 1_7 GR promoter for demethylation. Transcription factors were shown before to target demethylation to specific sequences. A good example is the involvement of NF kappa B in demethylation of immunoglobulin kappa light chain gene enhancer, which requires the presence of a *cis* acting recognition element for this transcription factor (Kirillov et al., 1996), and demethylation mediated by the maize suppressor-mutator transposon-encoded TnpA protein (Dahlman-Wright et al., 1991). We have previously shown that pharmacological stimulation of histone acetylation by TSA induces replication-independent demethylation in cell culture (Cervoni and Szyf, 2001) and we proposed that transcription factors trigger demethylation by recruiting HATs to promoter. We therefore hypothesized that NGFI-A targets the exon 1_7 GR promoter for demethylation during days 1 to 6 after birth in offspring of High LG-ABN by recruiting CBP to the promoter. The notion that demethylation of exon 1_7 GR promoter is stimulated by histone acetylation is supported by our previous data showing that pharmacological hyperacetylation of exon 1_7 GR promoter induced by TSA results in demethylation of this gene in the hippocampi of adult offspring of Low LG-ABN mothers (see Chapter 6).

For NGFI-A to be involved in targeting demethylation of the exon 1_7 sequence during early postnatal development, the transcription factor has to interact with the methylated GR promoter. Although we show here that the interaction of NGFI-A with exon 1_7 GR promoter is significantly diminished by DNA methylation, ectopic expression of NGFI-A results in some binding to *in vitro* methylated exon 1_7 GR promoter reporter and triggers its demethylation. Because the non-integrated plasmid does not bear an origin of replication and does not replicate in HEK cells, the assay measures the effects of NGFI-A on active, replication independent demethylation, which is consistent with the replication-independent demethylation seen in neuronal cultures. This demethylation triggered by NGFI-A is dependent on interaction between NGFI-A and its cognate response element in the exon 1_7 sequence since mutation of the 3′ CpG dinucleotide that abolishes interaction of NGFI-A with the GR promoter also abolishes demethylation. This rules out the possibility that NGFI-A acts indirectly by activating a different gene, which triggers demethylation of the exon 1_7 sequence that then enables interaction of NGFI-A with the unmethylated GR promoter. If interaction of NGFI-A with its target results in demethylation, then NGFI-A bound exon 1_7 GR promoters should be enriched for demethylated sequences which is what is observed both *in vivo* and in transiently transfected cells in culture by sodium bisulfite mapping of ChIPed material with anti NGFI-A antibody. The involvement of NGFI-A is further supported by the inhibition of exon 1_7 GR promoter demethylation in hippocampal neuronal culture following NGFI-A antisense treatment. Thus, we propose a dual role for NGFI-A in activation and demethylation of exon 1_7 GR promoter. High levels of NGFI-A target demethylation activity to exon 1_7 sequence, while normal levels of NGFI-A expressed in adult brain at normal levels act as a methylation dependent regulator of the GR promoter. The inhibition of NGFI-A binding by methylation of the 5′ CpG dinucleotide results in selective activation of unmethylated exon 1_7 GR promoters.

Concluding remarks

The function of such immediate early genes provides is a mechanism by which the environment may interact with the genome to shape neuronal function. Examination of immediate early genes from a developmental perspective will provide information on how the environment shapes phenotypic behaviour.

Chapter 10
General Discussion

A common theme across all species is that exposure of the mother to environmental adversity alters the nature of mother-offspring interaction, which, in turn, influences the development of defensive responses to threat as well as reproductive strategies in the progeny (Agrawal, 2001; Rossiter, 1999). Environmental effects on the development of individual differences in defensive reactions to threat or reproductive strategies represent adaptations, or developmental strategies, that serve to refine the function of various organs to an anticipated level of environmental demand. The effects of environmental quality on development are mediated by variations in parental investment, such as nutrient supply provided by the parent as well as behavioural interactions. Variations in parental investment in mammals commonly involve the quantity and quality of behavioural interactions between parent and offspring. The actual form of the variation in parental investment will vary, but the effects on phenotypic plasticity are common and in each case affect the development of (1) defensive responses and (2) reproductive strategies. Indeed, across a large range of species, variations in parental investment are associated with phenotypic plasticity in defensive and reproductive systems. Such that environmental adversity alters parent-offspring interactions and that, these effects serve as the basis for phenotypic plasticity. The variations in parent-offspring interactions are, in effect, the forecast of environmental quality for the offspring. However, how are these parental effects rendered permanent throughout the life of the offspring?

In the rat, the mechanism for such transgenerational effects appears to involve variations in maternal LG-ABN behaviour which influence hippocampal gene expression and physiological function in the offspring (Denenberg, 1999; Ottinger and Tanabe, 1969;

Ressler and Anderson, 1973). Importantly, the adult offspring of High LG-ABN mothers show significantly greater expression of hippocampal neurone survival markers NGF, BDNF and NR_1 and have lower levels of circulating GCs in comparison to the adult offspring of Low LG-ABN dams (Caldji et al., 1998; Liu et al., 2000; Liu et al., 1997). These findings, taken together with the maternal effects on hippocampal GR sensitivity, suggest a relation between maternal care, GC sensitivity and neurone survival in the developing rat hippocampus. However, though maternal care and synaptic survival in the neonatal rat hippocampus is associated with the expression of neurotrophic factors, there is little understanding of the underlying pathways involved in the preservation of maternal-care-driven individual differences in cognitive development, or even how maternal care influences neuronal survival during adulthood.

Molecular mechanisms for maternal effects on hippocampal neurone survival

Chronically high levels of circulating GCs in human populations are associated with mood disorders, memory deficits and hippocampal aging. During classical hippocampal neuronal death through ischemia, cells deprived of O_2 and glucose rapidly lose ATP and become depolarised, leading to the synaptic release of glutamate, accompanied by the electrogenic transport of glutamate from depolarised astrocytes (Nicholls and Attwell, 1990). The increase of extracellular glutamate results in over-stimulation of α-amino-3-hydroxt-5-methyl-4-isoxazole propionic acid (AMPA)-, kainate- and NMDA-type glutamate receptors, with consequent influx of Na^+ and Ca^{2+} ions. In parallel, GCs increase resting levels of intracellular Ca^{2+} by retarding removal of Ca^{2+} (Elliott and Sapolsky, 1992; Elliott and Sapolsky, 1993; Virgin et al., 1991). Indeed, NMDA receptors are triggered secondarily by Na^+ influx through AMPA-, kainate-, and NMDA-receptor-gated channels, by the activation of voltage-gated Ca^{2+} channels and reverse operation of the Na^+ and Ca^{2+} exchanger (Dirnagl et al., 1999). In summary, excessive Ca^{2+} influx through GC over-activity, severely compromise the hippocampal neurone's normal homeostatic cell-signalling pathways, resulting in cell death by either necrosis (passive cell death) or apoptosis (active cell death) (Choi, 1995).

Several factors may determine which process predominates. The rheostat model states that the balance between death-promoting and death-repressing members of the BCL-

2 family determines whether a cell will undergo an apoptotic program after receiving a death stimulus (Oltvai and Korsmeyer, 1994; Oltvai et al., 1993). Results from *in vivo* studies within this thesis suggest that maternal effects on hippocampal neuronal survival are mediated by increases in hippocampal expression of the pro-apoptosis protein BAX (Figure 15) and increases in DNA fragmentation in offspring of Low LG-ABN mothers (Figure 16). BCL-2 family members are crucial for neuronal survival during development (Merry et al., 1994; Nijhawan et al., 2000). For example, postnatal BCL-2 knockout mice have significant degeneration of central motor neurones, sensory neurones, and sympathetic neurones (Michaelidis et al., 1996). Conversely, ectopic expression of BCL-2 in neurones results in brain hypertrophy (Farlie et al., 1995; Martinou et al., 1994). Deletion of BAX results in an increase in central motor neurones and more superior cervical ganglion neurones, with a gene dosing effect (Deckwerth et al., 1996). Furthermore, dephosphorylated BAD translocates to the mitochondria and is able to initiate apoptosis via heterodimerization with BCL-2. However, extracellular survival signals inhibit apoptosis by activating the phosphotydylinositol-3 kinase/Kat pathway, leading to BAD phosphorylation. Phosphorylated BAD binds 14-3-3 protein and is sequestered in the cytoplasm, resulting in cell survival (Zha et al., 1996). However, Ca^{2+} may also induce apoptosis by activating the calcineurin-dependent phosphatase that dephosphorylates BAD, leading to programmed cell death (Wang et al., 1999). Additionally, BAD interaction with BCL-2 can even displace BAX from the heterodimer. However, BCL-2 appears to actively inhibit the intracellular accumulation of Ca^{2+} following cell death induction, which could swing the pathway towards cell survival, depending on BCL-2 availability (Marin et al., 1996). Moreover, in BAX-deficient synaptic neurones there is a delay in Cyto-C release when NGF is removed, implying BAX catalyses apoptotic induction on withdrawal of NGF, as suggested in the neurotrophin model (Deshmukh and Johnson, 1998; Easton et al., 1997; Neame et al., 1998). Therefore, differences in BAX expression may be crucial for their involvement in developmental pruning of the hippocampus, involving intrinsic survival signals transmitted to the mitochondria from different cellular compartments, such as following tactile stimulation by maternal behaviour.

These studies suggest that maternal care influences neurone survival and hippocampal development in the rat. Prolonged exposure to elevated levels of GCs reduces hippo-

campal cell number (Sapolsky et al., 1985) and can induce cultured neurons to undergo apoptosis (Reagan and McEwen, 1997). The effects of GCs on stress-related neuronal death in the hippocampus are elicited, in part, through GR activity. Importantly, the nature of mother-offspring interactions influences hippocampal GR gene expression and the development of behavioural responses to stress in the offspring, which remain stable from early development to the later stages of life. A proposed mechanism for such long-term programming of HPA responses to stress and hippocampal GR function is described below.

Molecular mechanisms for maternal effects on HPA responses to stress

Results from *in vitro* and *in vivo* studies shown here suggest that maternal effects on hippocampal GR expression are mediated by increases in 5-HT turnover and in hippocampal expression of the transcription factor NGFI-A (Figure 10). *In vitro*, 5-HT increases expression of both GR and NGFI-A in cultured rat hippocampal neurons, and the effect of 5-HT is blocked by concurrent treatment with an antisense oligonucleotide directed to NGFI-A mRNA (Figure 38). The 5′ non-coding variable exon 1 region of the rat hippocampal GR gene (Figure 14) contains multiple alternate sequences, including the exon 1_7 sequence, which appears to be a brain-specific promoter (McCormick et al., 2000). In adult rats, hippocampal expression of GR mRNA splice variants containing exon 1_7 is increased by postnatal handling or maternal LG-ABN. Exon 1_7 contains an NGFI-A-binding consensus sequence. Maternal LG-ABN increases hippocampal NGFI-A expression, and ChIP assays with hippocampal samples from PND 6 pups reveal that NGFI-A binding to the exon 1_7 GR promoter is dramatically increased in the offspring of High LG-ABN mothers compared with the offspring of Low LG-ABN mothers (Figure 35). Co-transfection of a NGFI-A vector and an exon 1_7-luciferase construct into HEK cells increases luciferase activity (Figure 39), reflecting NGFI-A-induced activation of transcription through the exon 1_7 GR promoter. These findings suggest that NGFI-A might increase GR expression in hippocampal neurons and provide a mechanism for the effect of maternal care over the first week of life. However, although there are striking differences in NGFI-A expression in the neonatal offspring of High LG-ABN and Low LG-ABN mothers, there is no effect of maternal care on hippocampal NGFI-A expression in the adult [(Meaney et al., 2000) and data not shown]. This begs the question of whether the increased NGFI-A and exon 1_7 interaction in the PND 6 pups of High LG-ABN mothers result in an epigenetic modification of the

exon 1_7 GR promoter sequence that alters NGFI-A binding and maintains the maternal effect into adulthood.

Epigenetic programming of HPA stress responses

We initially found evidence for greater methylation across the exon 1_7 GR promoter sequence in the hippocampus in adult offspring of Low LG-ABN mothers than in those of High LG-ABN mothers (Figure 17). These findings suggested maternal effects on DNA methylation patterns on the offspring. We then examined the methylation status of individual CpG dinucleotides in the exon 1_7 GR promoter sequence using sodium bisulfite mapping, focusing on the NGFI-A response element (Figure 17). The results reveal significant differences in cytosine methylation in the 5′ CpG dinucleotide within the NGFI-A response element. The 5′ CpG dinucleotide appears to be always methylated in the offspring Low LG-ABN mothers, and rarely methylated in those of High LG-ABN dams. Cross-fostering reverses the differences in the methylation of the 5′ CpG dinucleotide and suggests a direct relationship between maternal behaviour and changes in DNA methylation of the exon 1_7 GR promoter (Figure 18). The effect of maternal care is remarkably specific, with highly significant alterations in the methylation status of the 5′ CpG dinucleotide but no effect on the 3′ CpG dinucleotide. The difference in methylation within the 5′ CpG dinucleotide of the NGFI-A response element suggests alteration of NGFI-A binding. *In vitro* binding of increasing concentrations of purified recombinant NGFI-A protein to the NGFI-A response element was examined using an EMSA with ^{32}P-labelled synthetic oligonucleotide sequences bearing the NGFI-A-binding site differentially methylated at the 5′ or 3′ CpG dinucleotides. The results indicate that methylation of the cytosine within the 5′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 GR promoter inhibits NGFI-A binding (Figure 37), a finding consistent with the role for cytosine methylation. Differences in NGFI-A expression between the offspring of High and Low LG-ABN mothers in early postnatal life are no longer apparent in adulthood (Figure 34). Instead, it appears that the methylation of the NGFI-A response element interferes with NGFI-A binding to the exon 1_7 GR promoter in the offspring of Low LG-ABN mothers. Indeed, ChIP assays indicate that binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter in PND 6 pups is three-fold greater in offspring of High LG-ABN mothers than offspring of Low LG-

ABN dams (Figure 35). NGFI-A activates the genes by recruiting CBP (a HAT) to the GR promoter. Studies using the same tissue samples and an antibody against the acetylated form of histone H3 show increased association of acetylated histone H3-K9 with the exon 1_7 GR promoter in offspring of the High LG-ABN mothers (Figure 35). Because histone acetylation is associated with active states of gene expression, these findings are consistent with the idea of increased NGFI-A binding to the exon 1_7 GR promoter, recruiting HATs resulting in increased transcriptional activation. Moreover, transient transfection studies show that DNA methylation reduces the ability of NGFI-A to activate the exon 1_7 GR promoter in HEK cells (Figure 41). Taken together these findings suggest that an epigenetic positional modification at a single cytosine residue within the NGFI-A response element alters NGFI-A binding and might explain the sustained effect of maternal care on hippocampal GR expression and HPA responses to stress.

Reversal of maternal effects on GR expression and HPA stress responses

The fundamental difference between epigenetic and genetic modifications is the potential reversibility of epigenetic modifications. The possibility that certain environmental programming of behaviour is potentially reversible has immense therapeutic and social implications. Since neurons do not divide, epigenetic marks in the brain could be reversible only if the demethylation and methylation machinery is still present in the cell. Szyf has previously proposed that the DNA methylation pattern is maintained through life by a dynamic equilibrium of methylation and demethylation (Szyf, 2001). If this is true, it should be possible to reverse epigenetic marks by tilting the balance of the equilibrium using different pharmacological manipulations. In this thesis, I tested the possibility that the GR state of methylation could be shifted both ways by the appropriate pharmacological manipulations. As discussed previously (Section 2.5) HDAC inhibitors can trigger active, replication-independent DNA demethylation. Although the mechanism of this activation is unclear it has been proposed that increased acetylation results in increased accessibility of a gene to the demethylation machinery. We tested whether this approach could reverse the epigenetic state of GR in the adult offspring of Low LG-ABN mothers. Central infusion of adult rats with the HDAC inhibitor TSA significantly increased histone H3-K9 acetylation (Figure 21), cytosine demethylation (Figure 22) and NGFI-A binding (Figure 21) to the exon 1_7 promoter in the offspring of Low LG-ABN mothers to levels comparable with

those observed in the offspring of High LG-ABN dams. Expression profiling of TSA-treated rats reveal specific effects of TSA on the hippocampal transcriptome (Figure 31). The enhanced NGFI-A binding to the exon 1_7 promoter is associated with increased hippocampal GR expression in the offspring of Low LG-ABN mothers, to levels that are indistinguishable from those of the High LG-ABN offspring (Figure 23). More importantly, TSA infusion also eliminates the effect of maternal care on HPA responses to acute stress (Figure 24). Because there is considerable histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR promoter in the vehicle-treated offspring of High LG-ABN mothers, TSA is presumably less likely to effect NGFI-A binding and GR expression in these animals (Figure 21). These results suggest causal relationships between maternal care, histone acetylation, DNA methylation of the exon 1_7 GR promoter, GR expression and HPA responses to stress. These findings, suggest that DNA methylation patterns are dynamic and potentially reversible even in adult neurons, which presumably contain the machinery required to demethylate genes. We argued that if DNA methylating and demethylating enzymes dynamically maintain the DNA methylation pattern in adult neurons, then it should also be possible to reverse the demethylated state of the exon 1_7 GR promoter. Indeed, chronic central infusion of adult offspring of High or Low LG-ABN mothers with the methyl-donor L-methionine increases DNA methylation within the NGFI-A response element (Figure 25) and reduces NGFI-A binding to the exon 1_7 GR promoter (Figure 26) selectively in the offspring of High LG-ABN dams. These effects eliminate group differences in both hippocampal GR expression and HPA responses to stress (Figure 27). Methionine increases the levels of SAM and DNA methylation (Tremolizzo et al., 2002), and SAM could increase DNA methylation either by stimulating DNA methylation enzymes (Pascale et al., 1991) or by inhibiting demethylases (Szyf, 2001).

How does maternal care alter cytosine methylation?

The developmental studies shown here provide evidence for an active process of demethylation driven by maternal care. High and Low LG-ABN mothers differ in the frequency of pup LG-ABN only during the first postnatal week (Section 2.1.3). This period corresponds to appearance of the difference in DNA methylation of the NGFI-A response element of the exon 1_7 GR promoter (Figure 19). On ED 20 (i.e., 24-48 h before birth), the entire exon 1_7 region of the GR promoter is completely unmethylated. On PND 1 both the 5′ and 3′ CpG

dinucleotides within the NGFI-A response element are heavily methylated in both the High and Low LG-ABN offspring, suggesting a comparable postnatal wave of *de novo* methylation. The differences in methylation emerge between PND 1 and 6, which is precisely when differences in the maternal behaviour of High and Low LG-ABN dams are apparent. By PND 6 the 5′ CpG dinucleotide of the NGFI-A-response element is demethylated in the High LG-ABN but not in the Low LG-ABN group, and the maternal effect persists through to adulthood. These findings suggest that the group difference in DNA methylation involves a process of demethylation. We are left with the question of how maternal care might activate demethylation of the exon 1_7 GR promoter. Note that on PND 1 the CpG dinucleotides of the NGFI-A consensus sequence in the exon 1_7 GR promoter are heavily methylated and, thus, in a low binding affinity state in the offspring of both High and Low LG-ABN mothers (Figure 19). We suggest that increased pup LG-ABN increases NGFI-A transcription by activating 5-HT_7 receptor signalling through cAMP and PKA (Figure 10). Increased NGFI-A levels enhance NGFI-A binding to the methylated exon 1_7 GR promoter, recruitment of HATs, histone acetylation and accessibility of demethylating enzyme(s) to the exon 1_7 GR promoter, resulting in DNA demethylation. Thus, NGFIA plays a dual role in regulation of GR expression. During early development, high levels of NGFI-A induced by maternal care interact with methylated GR and trigger its demethylation whereas later in life, physiological levels of NGFI-A discriminate between methylated and unmethylated GR promotes and selectively activate unmethylated GRs. The differential state of methylation of the exon 1_7 sequence in offspring of High and Low LG-ABN results in different levels of GR promoter firing.

The causal relationship between environmental stimulation, transcription factor activation and histone acetylation has been established using hippocampal neurons (Crosio et al., 2003). However, these previous experiment did not study the consequences of these manipulations to the state of covalent modification of the genome by DNA methylation. This model also assumes a steady state of DNA methylation and demethylation, the direction of which is determined by the state of chromatin structure (Section 2.5). The hypothesis predicts that both DNA methyltransferases and demethylases are present in neurons and that alterations of the chromatin state by persistent physiological signals can alter the methylation of a gene in post-mitotic tissue such as hippocampal neurons. Pharmacological

activation of chromatin structure by HDAC inhibitors, such as TSA, can trigger replication-independent, active demethylation of DNA. As described above, TSA treatment of adult offspring of Low LG-ABN mothers increases histone H3-K9 acetylation and NGFI-A binding at the exon 1_7 GR promoter (Figure 21); predictably, TSA treatment also results in demethylation of the 5′ CpG dinucleotide (Figure 22) even in the absence of cell replication. We established the causal relation between NGFI-A binding, GR demethylation and expression using two tissue culture systems. In HEK cells transiently transfected with a methylated exon 1_7 GR promoter-luciferase vector, NGFI-A overexpression ultimately leads to demethylation of the 5′ CpG dinucleotide within the NGFI-A response element on the exon 1_7 construct. Because the non-integrated plasmid does not bear a human origin of replication and thus does not replicate in HEK cells, the assay measures the effects of NGFI-A on active, replication independent demethylation. To demonstrate that this DNA demethylation requires direct contact between NGFI-A and its recognition element, we performed site directed mutagenesis of the 5′ and 3′ CpG dinucleotides of the NGFI-A response element. Mutation of the 3′ CpG dinucleotide blocks the ability of NGFI-A to bind the GR promoter (Figure 41), and abolishes the ability of NGFI-A to demethylate (Figure 42) and activate (Figure 40) the exon 1_7 GR promoter. This data demonstrates that physical interaction between NGFI-A and the methylated GR promoter is required for NGFI-A to target demethylation. Bisulfite mapping confirmed that GR promoter sequences that interact with NGFI-A are demethylated.

Finally, hippocampal cell cultures treated with either 5-HT or 8-bromo-cAMP show increased GR expression and hypomethylation of the 5′ CpG dinucleotide within the NGFI-A response element on the exon 1_7 GR promoter, with no effect at the 3′ CpG dinucleotide (Figure 38), again in the absence of cell replication. Cultures maintained under control conditions show complete methylation of both the 5′ and 3′ CpG dinucleotides with the NGFI-A response element. The cultures were treated with mitotic inhibitors to prevent glial proliferation and labelling of newly generated cells by bromodeoxyuridine (BrdU) revealed little or no cell replication in the cultures at the time of 5-HT treatment. We are left with the question of how does NGFI-A trigger demethylation upon binding to specific sequences. NGFI-A can actively target methylated-DNA-binding proteins to genomic tar-

gets (Carvin et al., 2003). Interestingly, 5-HT increases expression of CBP in hippocampal cell cultures [and in Aplysia during conditioning (Guan et al., 2002)], and CBP binding to the exon 1_7 GR promoter is increased in the neonatal offspring of High LG-ABN mothers (Figure 35). Thus, one possibility is that NGFI-A recruits a CBP that increases acetylation and accessibility to demethylase and stable promoter activation (Figure 43). I have shown in this thesis that a pharmacological increase in acetylation *in vivo* results in demethylation of GR promoter in the hippocampus and it has been previously shown that increased acetylation targets demethylation of methylated sequences (Cervoni and Szyf, 2001).

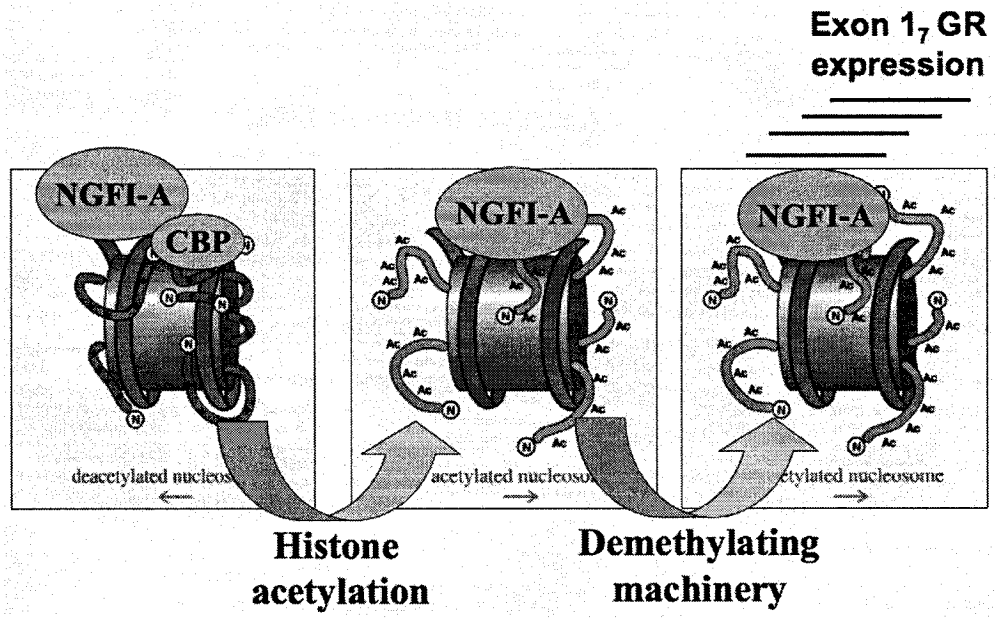

Figure 43: Regulation of GR gene expression. NGFI-A recruits a CBP that increases acetylation and accessibility to demethylase and stable GR promoter activation.

Revisiting phenotypic plasticity at the level of chromatin structure

A defining question for scientists interested in development concerns the mechanism by which environmental effects, including social interactions such as parenting, in early development are biologically embedded and thus sustained into adulthood. The challenge is to define the exact nature of the gene-environment interactions that mediate such environmental programming. The differential epigenetic status of the exon 1_7 GR promoter in the offspring of High LG-ABN mothers described here is a candidate paradigm for the mechanisms mediating the maternal effects on hippocampal gene expression. However, these findings are restricted to the study of a single promoter in only one gene in one brain re-

gion; at this time, these results might be best thought of as a proof of principle. The degree to which such results generalize to other instances of environmental programming remains to be determined, and could reveal alternative mechanisms for programming of the epigenome.

In the context of the present model (Figure 43), further studies are required to determine precisely how maternal behaviour alters epigenomic status of the GR exon 1_7 promoter. The data suggest an active process of demethylation. The findings discussed here, taken together with a recent publication on altered expression of IL-2 expression by T lymphocytes following activation (Bruniquel and Schwartz, 2003), implicate an active process of demethylation in non-transformed somatic cells. Active demethylation now appears to be an accepted event (Bird, 2003). There is evidence for existence of a demethylase (Bhattacharya et al., 1999; Ramchandani et al., 1999), although the exact identity of the demethylase responsible in these studies remains to be clarified. Nevertheless, these findings provide a possible mechanism for the environmental programming of gene expression and function during development and beyond. Studies on the reversal of maternal effects on DNA methylation using either TSA or methionine suggest that neurones express the enzymatic machinery necessary for methylation and demethylation in adulthood. DNA methylation, although a stable epigenetic mark maintained through carbon-carbon bonds, can be altered through sustained alterations of chromatin structure such as histone acetylation. These findings thus raise the fascinating question of the degree to which such processes might remain sensitive to environmental regulation throughout life. It is important to note that maternal effects on the expression of defensive responses, such as increased HPA activity, are a common theme in biology (Mousseau and Fox, 1998). Such effects could reflect environmental experience of the mother that is translated, through an epigenetic mechanism of inheritance, into phenotypic variation in the offspring. Indeed, maternal effects could result in the transmission of adaptive responses across generations. In humans, such effects might contribute to the familial transmission of risk and resilience. Accordingly, it is interesting to consider the possibility that epigenetic changes could be an intermediate process that imprints dynamic environmental experiences on the fixed genome, resulting in stable alterations in phenotype; i.e., by a process of experience dependent-chromatin plasticity.

A natural model for neurodegenerative diseases with underlying epigenetic disregulation

Studies within this thesis examined the effects of TSA and methionine treatment on brain gene expression and physiological and behavioural responses to stress within the context of early life experience. Microarray analysis revealed that TSA and methionine treatment altered the expression of several genes, similar to those observed in human neurodegeneration and dementia. For example, enhanced expression levels of vof-16 within the hippocampus are associated with progressive cognitive deficits, neuronal damage and impairment of learning and memory performance (Tohda and Watanabe, 2004). Furthermore, hippocampal ATRX dysregulation is associated with numerous forms of syndromal X-linked mental retardation including, Carpenter syndrome, Juberg-Marsidi syndrome and Smith-Fineman-Myers syndrome. Accordingly, TSA and a variety of compounds with a similar function are currently used as clinical therapeutics for the regulation of diseases caused by aberrant gene expression (Figure 44).

	Substance groups	Derivatives
HDAC inhibitors	Short chain fatty acids	Butyrate, valproate
	Hydroxamates	Trichostatin A (TSA), oxamflatin, scriptaid, suberoylanilide hydroxamic acid (SAHA), pyroxamide, NVP-LAQ824, cyclic hydroxamic acid containing peptides (CHAPs)
	Cyclic tetrapeptides	Trapoxins, HC-toxin, chlamydocin, apicidin, depsipeptide (FR901228 or FK228)
	Benzamides	MS-275, N-acetyldinaline (CI-994)
	Sulfonamide anilides	N-2-aminophenyl-3-[4-(4-methyl benzenesulfonylamino)-phenyl]-2-propenamide
	Others	Depudecin
Demethylating agents	Cytidine analogs	5-Azacytidine (azacytidine), 5-aza-2'-deoxycytidine (decitabine), arabinofuranosyl-5-azacytosine (fazarabine)

Figure 44: Overview of HDAC inhibitors and demethylating agents frequently used for clinical and research purposes [adapted from (Mei et al., 2004)].

Methionine participates in a wide range of biochemical reactions including the production of the methyl-donor SAM, which serves as the donor of methyl groups for DNA methylation. In the rat, the physiological blood plasma methionine concentration range is 5.05 ± 1.14 μmol/L (Durand et al., 1996). We centrally infused 100 μg/ml of methionine into the rat brain. As best as we are able to estimate, with the actual volume of the lateral

ventricle being the obstacle, our infusions (0.5 μg methionine), produced a local concentration of 10 μMol, and thus a concentration that is within the physiological region. Intracellular SAM levels can also be altered through folate supplementation (Miller et al., 1994; Poirier et al., 2001). These findings together with our methionine data raise the possibility that diet could modify epigenetic programming in the brain not only during early development but also in adult life. This could have important therapeutic implications, since aberrant DNA methylation is involved in neurological disease such as fragile X syndrome and is potentially associated with multiple psychiatric and behavioural conditions including schizophrenia (Grayson et al., 2005). Indeed, SAM is currently used in the treatment of certain forms of clinical depression (Cantoni, 1975; Fazio et al., 1973; Mudd and Cantoni, 1958). The suggested mechanisms of action of SAM in depression include increases in GABA-ergic tone (e.g., $GABA_B$ receptor expression) and increases in SAM-dependent methylation (e.g., methylbarinine). However, antidepressants also influence central monoaminergic function (e.g., 5-HT activity), which regulates hippocampal GR expression and so subsequently effect HPA responses to stress. Brain methionine levels are also possibly involved in the expression of gender-related aggressive behaviour. The $GABA_A$ receptor agonist 3α-hydroxy-5α-pregnan-20-one (3α-5α-P, allopregnanolone, Allo) enhances noradrenalin release in the ventral noradrenergic pathway, resulting in an increase in aggressive behaviour. Methionine treatment results in a down regulation of Allo expression and aggressive behavioural response (Pinna et al., 2004). The histone deacetylase inhibitor valproate (Figure 44) reverses this process, suggesting that, together with our studies using TSA, histone tail acetylation reverses the action of methionine on DNA methylation.

Increases of methionine within the brain decreases GABA synthesis enzyme L-glutamate-1-carboxylyase (glutamate decarboxylase, GAD) isoform GAD67 and reelin mRNA expression (Tremolizzo et al., 2002). Our microarray studies and RT-PCR analysis using rat hippocampal tissue revealed a naturally occurring difference in reelin expression within the adult offspring as a function of early life experience (Figure 33). In humans, autosomal recessive lissencephaly with cerebellar hypoplasia, accompanied by severe delay, hypotonia and seizures, has been associated with mutations of the reelin gene. Furthermore, dysregulation of GABA-ergic and reelin systems are vulnerability factors for the aetiology and pathophysiology of schizophrenia (Caruncho et al., 2004). Reelin is synthe-

sized and secreted into extracellular matrix by cortical GABA-ergic interneurones and binds with high affinity to the extracellular domain of integrin receptors expressed in dendritic shaft and spine postsynaptic densities (DSPSD) of pyramidal neurons within the hippocampus (Pesold et al., 1998). Dong et al. (2003) studied the effect of reelin on activity-regulated cytoskeletal protein (Arc) and total protein synthesis in synaptoneurosomes prepared from mouse neocortex. These studies showed that reelin binds with high affinity to integrin receptors expressed in synaptoneurosomes and thereby activates Arc protein synthesis (Dong et al., 2003). Therefore, reelin may have important implications in regulating developmental processes such as neuronal migration, dendritic sprouting, synaptogenesis, and axon pruning, as well as being involved in regulation of synaptic plasticity through life. Interestingly, we have previously shown that the adult offspring of High LG-ABN mothers show increased hippocampal synaptic density and perform better in paradigms that test hippocampal-dependent memory and learning, compared with the adult offspring of Low LG-ABN mothers (Bredy et al., 2003a; Bredy et al., 2003b; Liu et al., 2000). Therefore, the rat model of natural variations in maternal care could be potentially useful in the study of gene-environment-therapeutic interactions of genomic regions that are known to be involved in disease syndromes.

Methodological and therapeutic implications

The present thesis may also have important methodological implications on designing future basic research tools or clinical therapeutics. Indeed, the complexity of the CNS renders it vulnerable to a number of different diseases, often caused by only small variations in gene sequence or expression level. Thus, the ability to selectively regulate gene expression is of tremendous importance. Antisense strategies offer the possibility of investigating a single member of a closely related family of proteins that cannot be easily targeted using small-molecule pharmacological tools (Crooke, 2004). Antisense oligonucleotides base pair with mRNA and pre-mRNAs and can potentially interfere with several steps of RNA processing and message translation, including splicing, polyadenylation, export, stability and protein translation. In the studies described in this thesis, a chemically modified phosphorothioate antisense oligonucleotide was used to down regulate NGFI-A gene expression through triggering the degradation of NGFIA mRNA and pre-mRNA by RNaseH. The decease in hippocampal NGFI-A expression was accompanied by a reduction of hippocam-

pal GR expression. Equally, a similar type of approach could be used to down regulated transcription factors involved in neurodegeneration (for example, BAX expression). However, highly selective oligonucleotides could be more rapidly generated by RNA interference (RNAi) technology (Mello and Conte, 2004). A comparison of the mechanism of antisense and siRNA action is shown in Figure 45. Together, antisense oligonucleotides and siRNA-mediated therapies may hold great promise for the treatment of CNS diseases in which neurodegeneration is linked to overproduction of endogenous protein or to synthesis of aberrant proteins coded by dominant mutant alleles. Indeed, gene silencing by antisense oligonucleotides and siRNA has been demonstrated in neurones, and several targets involved in pain perception have already been addressed (Kurreck, 2004). Nevertheless, difficulties related to the crossing of the blood-brain barrier and antisense oligonucleotide efficacy remain a significant hindrance with this type of therapeutic approach (Xu and Juliano, 2005).

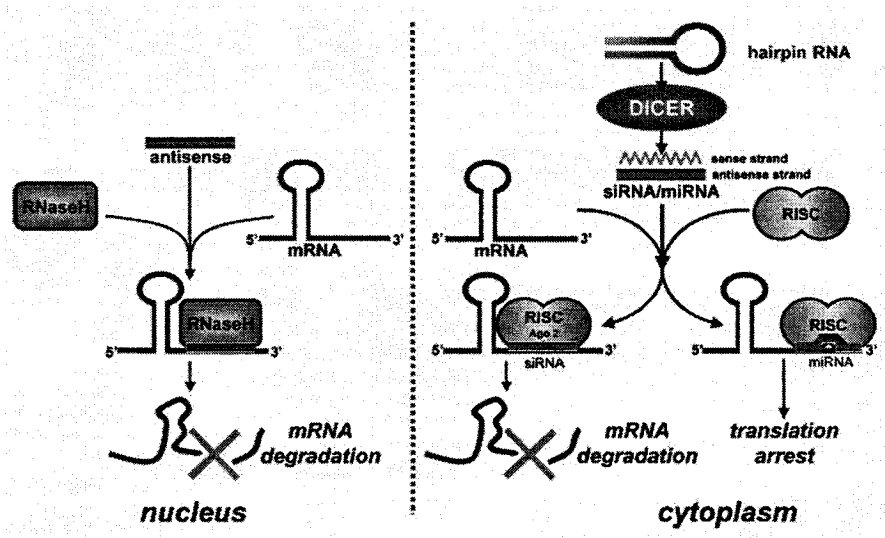

Figure 45: Mechanisms of antisense and siRNA action. Antisense oligonucleotides (left) are transfected into the cell. Within the cell's nucleus, a common mechanism of antisense action is RNaseH-mediated RNA degradation subsequent to the binding of antisense to its complement in the mRNA target. siRNA oligonucleotides (right) can also be delivered by transfection. Alternatively, hairpin RNAs can be expressed from plasmids or viral vectors (usually from pol III promoters) and are cleaved by the Dicer nuclease to siRNAs/miRNAs. Within the cell's cytoplasm, a single strand of the siRNA is loaded on to the RISC complex, where it can cleave a target its complement in the mRNA target. In addition, miRNA with partial mismatches can bind to an mRNA and cause inhibition of translation. Abbreviations denote the following: siRNA, Small interfering RNA; miRNA, microRNA; Ago 2, Argonaute 2 [adapted from (Juliano et al., 2005)].

The use of designed transcription factors has developed an interesting technology

that allows either the enhancement or the reduction of expression of a targeted gene (Pabo et al., 2001). Thus, a new approach would be to tailor a very efficient NGFI-A transcription factor that targets its cognate response element on the exon 1_7 GR promoter, which we have found to be critical in determining HPA responses to stress. NGFI-A is a member of the Zif transcription factor family, using the Cys2–His2 Zif motif for DNA-binding. The most recently designed transcription factors have polydactyl multi-Zif proteins that recognize long stretches of DNA, making them highly selective. For example, a 6-Zif protein recognizing 18 bases would theoretically be able to uniquely bind its target within a pool of almost 70 billion base pairs (Beerli and Barbas, 2002). The development of designed transcription factors is shown in Figure 46. Essentially, a designed NGFI-A-like protein would contain a DNA-binding domain responsible for the specific recognition of the exon 1_7 GR promoter and one or more effector domains to activate or repress transcription of hippocampal GR expression (Blancafort et al., 2004).

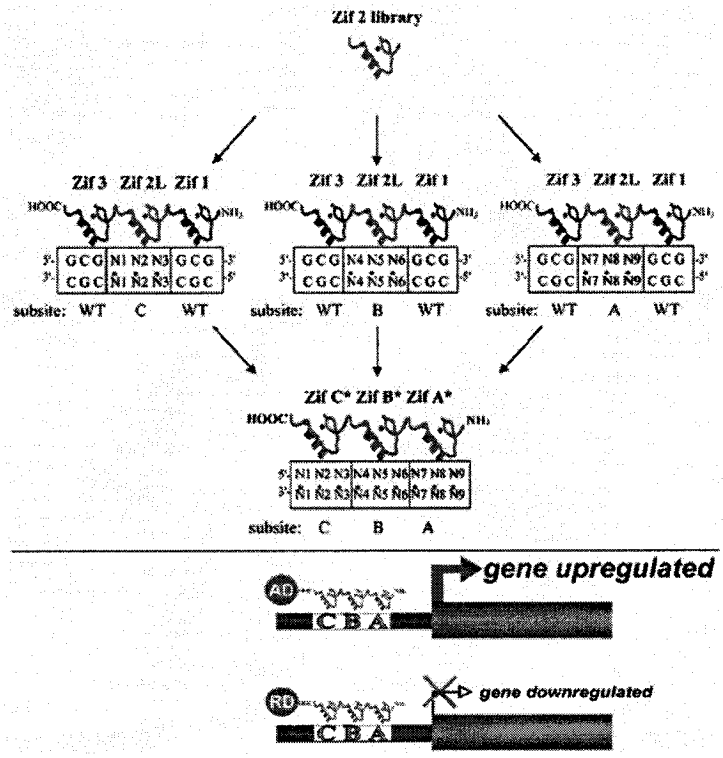

Figure 46: Development of designed transcription factors. Zif domains can be selected to bind to any desired DNA triplet using a combinatorial library strategy such as phage display. Individual Zifs can be combined to make multi-finger proteins that selectively bind to extended regions of DNA in the promoter regions of genes. The addition of transactivating or repressor domains allows positive or negative regulation of gene expression. Abbreviations denote the following: N1-N9, various

bases (N* is the complement); A-C, Zif triplets; Zif*, Zifs selected from the library screening; WT, wild type; AD, activation domain; RD, repressor domain [adapted from (Juliano et al., 2005)].

As mentioned before, GR expression is crucial for normal cell function and steroid resistance. Thus, the development of an NGFI-A-like protein could have significant implications for the treatment of allergic and autoimmune diseases that usually involve chronic steroid administration. Transfected cells would be thus engaged in relieving the immunosuppression. Furthermore, this approach could have significant implications for the treatment of other diseases including cancer. NGFI-A is over-expressed in prostate cancer, and its expression pattern suggests that NGFI-A could potentially regulate a number of steps involved in initiation and progression of the cancer, such as mitogenesis, invasiveness, angiogenesis and metastasis (Svaren et al., 2000). In this case, treatment with the dominant negative form of the NGFI-A-like protein may have some potential.

In summary, although there is no magic bullet for epigenetic gene regulation, approaches using antisense, siRNA and designed transcription factors may assist in the study of the role of individual genes within cellular processes and behavioural responses to stress and have the potential to be used in the treatment of stress-related illness and other diseases.

Future directions

Of course, there is a myriad of questions that remain unanswered: are there periods later in development (for example, during aging) that remain sensitive to epigenome modification by behavioural programming?; what other brain structures are involved in processing the effects of such behaviour?; how does maternal LG-ABN behaviour increase levels of T_3?; which thyroid and serotonergic circuits are crucial for the down stream hippocampal NGFI-A expression?; why and how does NGFI-A target the exon 1_7 GR promoter?; how is the DNA targeted for demethylation and what is the process of DNA demethylation?; is the methylation pattern of the exon 1_7 GR promoter tissue specific?; what are the expression profiles and epigenetic status of the remaining exon 1 GR putative promoters?; indeed, is the epigenetic status of other genes in different organs regulated in a similar manner by subtle changes in their environment? Of course, this list is by no means exhaustive. Accordingly, I would prefer to briefly describe two animal models that could potentially increase the general understanding of how epigenetic modulation of gene expression in

mammalian cells occurs through maternal programming.

The assumption is that licking and grooming, particularly of anogenital region, is the crucial facet of maternal care that programs the hippocampal GR epigenetic status and GC physiological function during brain development. However, other forms of maternal behaviour are potent sources of stimulation for the pup (for review see section 2.1). With this in mind, the neonates could be artificially reared using the pup-in-the-cup paradigm, which allows for experimental manipulation of brain development during the early postnatal brain growth spurt in the rat that coincides with that of the third trimester of human pregnancy (Gonzalez et al., 2001). In brief, the pups are removed from the nest and undergo a surgical procedure called a gastrostomy. The surgery involves inserting a leader wire sheathed in tubing into the pup's mouth and down the oesophagus and through the lateral wall of the stomach. Following the gastrostomy, the pups are housed individually in plastic cups, which float in temperature-controlled water bath. The cups contain bedding and the lids remain open to allow the gastrostomy tubing to emerge, and to connect to nearby syringes containing milk formula diet mounted on timer-controlled infusion pumps. Crucially, all artificially reared animals require a minimal stimulation twice a day (the required minimum) with a warm, wet paintbrush swiping their anogenital regions for 45 sec to stimulate urination and defecation. This stimulation manipulation is then carried out from the day the pups are placed on the pumps (PND 4) to the day of weaning (PND 20). On PND 20, the artificially reared animals are housed with a same-sex conspecific. Clearly, this procedure could easily be adapted for examining the quantitative and qualitative effects of tactile stimulation, hippocampal GC function and behavioural responses to stress. Firstly, the tactile stimulation could be titrated. Essentially, one would then be able measure the number of strokes required to demethylate a target gene sequence during the first week of life. Secondly, the milk formula could also be manipulated. This technique could be used to demonstrate temporal windows of vulnerability to GCs and for identifying dietary components contributing to metabolic adaptations in the pre-weaning period. Together, the ability to control temperature, titrate tactile stimulation and regulate diet could potentially provide a unique insight into epigenetic programming of endocrine, behavioural and emotional responses to stress in adult animals resulting from early life experience. Interestingly, a recent study examining the acute effects of postnatal touch stimulation re-

vealed that gentle brushing for 15 min per day during the first postnatal week of life induced a significant decrease (30-36 %) in serum GC secretion and an increase in GR expression within several brain regions, such as the hippocampus (19-21 %) and prefrontal cortex (26-34 %), in the neonatal pup (Jutapakdeegul et al., 2003).

Another question to address is the effects of both the prenatal and postnatal environment on the programming of GR expression in the neonate, which are critical in determining HPA responses to stress the adult animal. For nearly half a century inbred mouse strains have classically been used to search for the genes associated with a variety of traits including emotionality and stress-reactivity (Crabbe et al., 1999). Selective breeding and their persistence have supported the genetic basis of the differences between inbred strains even after postnatal cross-fostering. Inbred mouse strains demonstrate different levels of stress-reactivity. The C57BL/6NCr (C57) strain of mouse is classically considered to have an attenuated or dampened stress-response relative to the more excitable BALB/cAnNCr (BALB) strain of mouse, which is considered to be stress hyper-responsive (Bucan and Abel, 2002). The adult offspring of C57 compared with BALB mothers show increased hippocampal GR expression and enhanced GC feedback sensitivity. Predictably, adult offspring of C57 mothers show decreased hypothalamic CRH expression and more modest HPA responses to stress. Given that neurogenesis in the mouse occurs predominantly prenatally and given the abundant evidence for prenatal influences on subsequent development of the offspring it is likely that behavioural differences between inbred mouse strains might be a function of prenatal environment rather than a product of genetic differences between the offspring. A combination of prenatal and postnatal cross-fostering could be used to show whether both the prenatal and postnatal environments are critical in determining later adult behaviours of inbred mice (Francis et al., 2003). This effect would confirm and extend the findings of this thesis, which have demonstrated that some effects of the postnatal rearing environments can be transmitted to next generation through epigenetic programming by maternal behaviour. However, the mediating mechanism remains unclear. Given the homology between the rat and mouse exon 1_7 GR promoter, and the 100 % conserved CpG dinucleotides within the transcription factor consensus sequences across both species, it is likely that DNA methylation (which represents a stable epigenetic mark) may thus provide an explanation for the enduring effect on mother-infant interactions during prenatal

life on HPA responses to stress in the offspring. These experiments would demonstrate whether the stable behavioural differences between inbred strains are due to epigenetic factors, including prenatal environment in combination with postnatal rearing. Studies within this thesis using rats have demonstrated long-term, inter-generational consequences of individual differences in maternal care, providing a non-genomic mechanism for the transmission of behavioural traits. This research in mice would provide a unique insight into the nature of how the prenatal environment interacts with the postnatal environment to mark the genome for control gene expression and prime the developing pup to respond to postnatal care, such that a strain-specific phenotype develops independent of genotype (Francis et al., 2003).

Concluding remarks

In conclusion, the studies presented in this thesis provide support for the effect of maternal behaviour on hippocampal development, neurone survival and HPA responses to stress in the offspring, and that these effects are rendered permanent throughout life by epigenomic programming, however, reprogramming can take place at several points throughout the lifespan in response to changes in environmental conditions. Together, this work adds to the knowledge of how complex behaviour interacts with the epigenome and, in particular, illustrates the dynamic nature of gene-environment interactions throughout life. One hundred years following Pasteur's seminal studies on the effects of stress on immunity, we are beginning to understand the mechanisms whereby early-life experience suppresses or enhances expression of biological defence systems that respond to environmental adversity.

Chapter 11
Contributions to Original Knowledge

This thesis presents a thorough investigation of the mechanisms regulating the expression of individual differences of hippocampal GR function and behavioural HPA stress responses in the rat. Using an animal model, previously been shown to express natural variations hippocampal GR gene expression and HPA stress responses, we first evaluated whether maternal care effects hippocampal expression of cell survival markers in the adult offspring. We then established whether maternal care effects epigenomic programming of exon 1_7 GR promoter sequence and responses to stress in the adult offspring and investigated the mechanism by which developmental programming of the exon 1_7 GR promoter sequence occurs. The main conclusions from each study are listed in chronological order below.

11.1. *Effects of Maternal Care on Hippocampal Expression of BAX and Apoptosis*

- BAX, BAD and BCL-2 gene expression is detectable at the protein level in hippocampi from adult (PND 90) male offspring of High and Low LG-ABN dams.

- BAX protein levels are significantly greater in hippocampi from adult (PND 90) male offspring of Low LG-ABN dams.

- The number of cells undergoing apoptosis is significantly greater in hippocampi from adult (PND 90) male offspring of Low LG-ABN dams.

11.2. *Epigenetic Programming by Maternal Behaviour*

- The exon 1_7 glucocorticoid receptor promoter is significantly hypomethylated in hippo-

campi from adult (PND 90) male offspring of High LG-ABN dams.

- The cytosine residue within the 5′ CpG dinucleotide of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter is significantly hypomethylated in hippocampi from adult (PND 90) male offspring of High LG-ABN dams.

- The caring-mother is the predictor of the methylation status of the cytosine residue in the 5′ CpG dinucleotide of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter in hippocampi from adult (PND 90) male offspring.

- The exon 1_7 glucocorticoid receptor promoter is completely non-methylated in ED 20 hippocampi from offspring of High and Low LG-ABN dams.

- The exon 1_7 glucocorticoid receptor promoter is *de novo* methylated by PND 1 in hippocampi from offspring of High and Low LG-ABN dams.

- The cytosine residues within the 5′ and 3′ CpG dinucleotides of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter are *de novo* methylated to the same extent by PND 1 in hippocampi from offspring of High and Low LG-ABN dams.

- The cytosine residue within the 5′ CpG dinucleotide of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter is significantly demethylated by PND 6 in hippocampi from offspring of High LG-ABN dams.

- The cytosine residue within the 5′ CpG dinucleotide of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter remains significantly hypomethylated from PND 6 through to adulthood (PND 90) in hippocampi from offspring of High LG-ABN dams.

- The HDAC inhibitor, TSA, reverses the maternal effect on cytosine methylation, such that the exon 1_7 glucocorticoid receptor promoter is significantly demethylated in hippocampi from TSA (100 ng/ml)-treated adult male (PND 90) offspring of Low LG-ABN dams.

- The cytosine residue within the 5′ CpG dinucleotide of the NGFI-A binding site within

the exon 1_7 glucocorticoid receptor promoter is also significantly demethylated in hippocampi from TSA (100 ng/ml)-treated adult (PND 90) male offspring of Low LG-ABN dams.

- The exon 1_7 glucocorticoid receptor promoter is bound to acetylated histone H3-K9 and NGFI-A protein in hippocampi from adult male (PND 90) offspring of High and Low LG-ABN dams.

- The exon 1_7 glucocorticoid receptor promoter is bound to significantly greater levels of acetylated histone H3-K9 and NGFI-A protein in hippocampi from adult (PND 90) male offspring of High LG-ABN dams.

- TSA eliminates the maternal effect on histone acetylation and NGFI-A binding, such that the exon 1_7 glucocorticoid receptor promoter is bound to significantly increased levels of acetylated histone H3-K9 and NGFI-A protein in hippocampi from TSA (100 ng/ml)-treated adult (PND 90) male offspring of Low LG-ABN dams.

- Glucocorticoid receptor gene expression is detectable at the protein level in hippocampi from adult male (PND 90) offspring of High and Low LG-ABN dams.

- Glucocorticoid receptor protein levels are significantly greater in hippocampi from adult (PND 90) male offspring of High LG-ABN dams.

- TSA eliminates the maternal effect on glucocorticoid receptor expression, such that glucocorticoid receptor protein levels are significantly increased in hippocampi from TSA (100 ng/ml)-treated adult (PND 90) male offspring of Low LG-ABN dams.

- Acute restraint stress significantly increased HPA axis activation (i.e., as shown by increased plasma corticosterone levels) in the adult (PND 90) male offspring of High and Low LG-ABN dams.

- Plasma corticosterone levels are significantly greater in restraint-stressed adult (PND 90) male offspring of Low LG-ABN dams.

- TSA eliminates the maternal effect on HPA responses to stress, such that plasma corti-

costerone levels are significantly decreased in restraint-stressed, TSA (100 ng/ml)-treated, adult (PND 90) male offspring of Low LG-ABN dams.

11.3. *Reversal of Maternal Programming in Adulthood*

- The essential amino acid, L-methionine, reverses the maternal effect on cytosine methylation, such that the exon 1_7 glucocorticoid receptor promoter is significantly remethylated in hippocampi from methionine (100 μg/ml)-treated adult (PND 90) male offspring of High LG-ABN dams.

- The cytosine residue within the 5′ CpG dinucleotide of the NGFI-A binding site within the exon 1_7 glucocorticoid receptor promoter is also significantly remethylated in hippocampi from methionine (100 μg/ml)-treated adult (PND 90) male offspring of High LG-ABN dams.

- L-methionine eliminates the maternal effect on NGFI-A binding independent of histone acetylation, such that the exon 1_7 glucocorticoid receptor promoter is bound to significantly decreased levels of NGFI-A protein in hippocampi from methionine (100 μg/ml)-treated adult male (PND 90) offspring of High LG-ABN dams.

- L-methionine eliminates the maternal effect on glucocorticoid receptor expression, such that glucocorticoid receptor protein levels are significantly decreased in hippocampi from methionine (100 μg/ml)-treated adult (PND 90) male offspring of High LG-ABN dams.

- L-methionine eliminates the maternal effect on HPA responses to stress, such that plasma corticosterone levels are significantly increased in restraint-stressed, methionine (100 μg/ml)-treated adult male (PND 90) offspring of High LG-ABN dams.

- ICV infusion of TSA or methionine can eliminate the maternal effect on hippocampal GR expression and endocrine and behavioural responses to stress

- During the testing period of a forced-swim paradigm, the adult (PND 90) male offspring of Low LG-ABN mothers spend a significantly greater length of time immo-

bile in comparison to the offspring of High LG-ABN mothers.

- L-methionine eliminates the maternal effect on forced-swim performance, such that the length of time spent immobile during the testing period is significantly decreased in methionine (100 μg/ml)-treated, adult (PND 90) male offspring of High LG-ABN dams.

11.4. *Maternal Programming of the Hippocampal Transcriptome*

- Adult (PND 90) male offspring of High LG-ABN mothers are behaviourally less anxious/fearful than offspring of High LG-ABN dams, as observed in the length of time the animals spent exploring in an inner-field of an open-field arena.

- There are no significant differences between the overall open-field motor activities of adult (PND 90) male offspring of High and Low LG-ABN dams.

- TSA eliminates the maternal effect on open-field performance, such that the length of time spent in the inner-field is significantly increased in TSA (100 ng/ml)-treated, adult (PND 90) male offspring of Low LG-ABN dams.

- L-methionine eliminates the maternal effect on open-field performance, such that the length of time spent in the inner field is significantly decreased in methionine (100 μg/ml)-treated, adult (PND 90) male offspring of High LG-ABN dams.

- Affymetrix microarray analysis shows that over 50 % of hippocampal mRNA transcripts affected within each treatment group are uniquely responsive to either maternal care, TSA or methionine treatment.

- 303 genes showed differences in expression in adult (PND 90) animals, which was a consequence of maternal care early in postnatal life.

- 543 genes showed differences in expression in adult (PND 90) animals, which was a consequence of maternal care early in postnatal life and TSA treatment in adulthood.

- TSA has a broader impact on gene expression than early life maternal care, which is expected since TSA is a global inhibitor of histone deacetylases.

- 337 genes showed differences in expression in adult animals, which was a consequence of maternal care early in postnatal life and L-methionine treatment in adulthood (PND 90).

- L-methionine has a broader impact on suppression of gene expression than early life maternal care, as anticipated from a manipulation that increases DNA methylation and is known to result in gene silencing.

- Maternal care-, TSA- and methionine-regulated RNA transcripts are all involved in similar classes of cellular function, encoding protein products involved in general cellular metabolism and energy production; protein synthesis, trafficking and turnover; signal transduction; and cell adhesion molecules involved in neuronal connectivity, structural cellular components and synaptic components.

- Though both TSA treatment and High maternal LG-ABN behaviour affected genes from similar categories, TSA treatment suppressed expression of a collection of unique transcripts that were different to those that were suppressed in the offspring of High LG-ABN dams

- Though both methionine treatment and Low maternal LG-ABN behaviour affected genes from similar categories, methionine treatment suppressed expression of a collection of unique transcripts that were different to those that were suppressed in the offspring of Low LG-ABN dams

- High licking and grooming increased hippocampal ATRX and Reelin expression with no significant effect on Vof-16 expression; TSA treatment increased hippocampal ATRX and Vof-16 gene expression with no significant effect on Reelin expression; whereas, Low LG-ABN and methionine treatment decreased expression of all three genes.

11.5. *NGFI-A Mediated Epigenetic Programming by Maternal Behaviour*

- The exon 1_7 glucocorticoid receptor promoter is bound to significantly greater levels of CBP, acetylated histone H3-K9 and NGFI-A protein in hippocampi from neonatal (PND 6) male offspring of High LG-ABN dams.

- Although the exon 1_7 glucocorticoid receptor promoter is hypermethylated in hippocampi from neonatal (PND 6) male offspring Low LG-ABN mothers in comparison with offspring of High LG-ABN dams, the methylation status of the exon 1_7 promoter sequences that are bound to NGFI-A *in vivo* are equally unmethylated in both groups.

- Methylation of the cytosine within the 5′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 glucocorticoid receptor promoter inhibits the binding recombinant NGFI-A protein.

- A single treatment of either 5H-T (100 nM) or 8-bromo cAMP (10 mM) causes demethylation of the cytosine within the 5′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 promoter and a significant increase of glucocorticoid receptor expression in hippocampal cell cultures 4 days post-transfection.

- NGFI-A antisense (1 μM) oligonucleotide treatment eliminates the effects of 5H-T (100 nM) treatment on methylation of the cytosine within the 5′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 promoter and glucocorticoid receptor expression in hippocampal cell cultures.

- Recombinant NGFI-A overexpression significantly increases exon 1_7 glucocorticoid receptor promoter activation as well as CBP association, histone H3-K9 acetylation and NGFI-A binding with the exon 1_7 sequence in HEK 293 cells 72 h post-transfection.

- C to A mutation of the cytosine within the 3′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 glucocorticoid receptor promoter eliminates the effects of recombinant NGFI-A overexpression on luciferase gene induction as well as

CBP association, histone H3-K9 acetylation and NGFI-A binding with the glucocorticoid receptor promoter in HEK 293 cells.

- Recombinant NGFI-A overexpression significantly increases methylated exon 1_7 glucocorticoid receptor promoter activation as well as CBP association, histone H3-K9 acetylation and NGFI-A binding with the glucocorticoid receptor promoter in HEK 293 cells 72 h post-transfection.

- C to A mutation of the cytosine within the 3′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 sequence eliminates the effects of recombinant NGFI-A overexpression on glucocorticoid receptor promoter activation as well as CBP association, histone H3-K9 acetylation and NGFI-A binding with the methylated glucocorticoid receptor promoter in HEK 293 cells.

- Recombinant NGFI-A overexpression induces active demethylation of the exon 1_7 glucocorticoid receptor promoter in HEK 293 cells 72 h post-transfection.

- C to A mutation of the cytosine within the 3′ CpG dinucleotide in the NGFI-A response element on the exon 1_7 sequence eliminates the effects of recombinant NGFI-A overexpression on active demethylation of the glucocorticoid receptor promoter in HEK 293 cells

Chapter 12
Appendices

12.1. *Exon 1_7 GR Promoter Methylation Developmental Time-Line*

We used the sodium bisulfite mapping technique to precisely map the methylation status of the cytosines within the exon 1_7 GR promoter over multiple developmental time points (Figure 47 to Figure 51). Interestingly, the CpG dinucleotide (site 12) of the AP-1 consensus sequence within the exon 1_7 GR promoter is similarly hypomethylated in the High LG-ABN offspring by PND 6, and this hypomethylation is sustained into adulthood. The results of developmental time-line study are very intriguing. From PND 6, the methylation patterns for each of the 17 individual CpG sites within the exon 1_7 GR promoter do not all remain at the exactly same frequency for each developmental time-point (see Figure 47 to Figure 51). This is consistent with the model that methylation, like most (if not all) biological process, is in a constant flux, but is stably maintained through a dynamic equilibrium. The developmental time-line may also help explain why the effect of maternal care on the hippocampal GR gene activity is not easily reversed when a High LG-ABN offspring is cross-fostered to a Low LG-ABN mother, in comparison to the substantial increase in hippocampal GR gene activity observed when a Low LG-ABN offspring is cross-fostered to a High LG-ABN mother. DNA methylation is a thermodynamically slower process in comparison to active demethylation. Implying, that the hippocampal exon 1_7 GR promoter, within the Low LG-ABN mother's biological offspring, becomes striped of CpG methylation by activity-dependent processive demethylation, resulting from the intense tactile stimulation exerted towards the pups by the High LG-ABN foster mother. Whereas, the hippocampal exon 1_7 GR promoter, within the High LG-ABN mother's biological offspring, passively gains CpG methylation (assuming some loss of methylation through ma-

ternal care by the biological High LG-ABN parent prior to cross-fostering) through a lack of stimulation of an activity-dependent processive demethylation process by the Low LG-ABN adoptive parent. The fact that High and Low LG-ABN offspring differ on epigenomic control of gene activity leaves the reader to speculate upon why the Low LG-ABN offspring are so plastic in response to the foster, High LG-ABN mother.

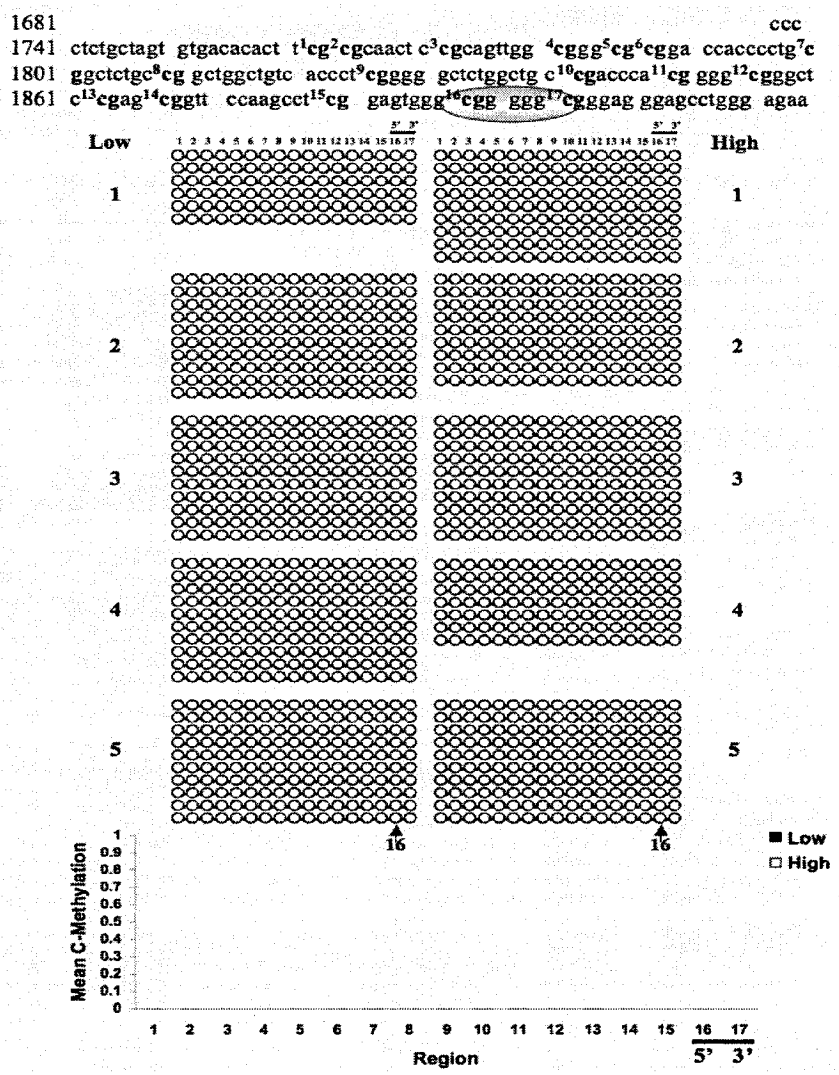

Figure 47: Methylation patterns of the exon 1_7 GR promoter in the hippocampi of embryonic day-20 High and Low LG-ABN offspring (n=5 animals/group). Top panel shows a sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding region (encircled). Middle panel shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 GR promoter region analyzed by sodium bisulfite mapping (6-10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 GR promoter region are labelled 1-to-17, highlighting the two CpG dinucleotides [site 16 (5') and site 17 (3')] within the NGFI-A binding region (black line). Black circles indicate methylated cytosines and white circles indicate non-methylated cytosines. The CpG dinucleotide at

site 16 (5') is again highlighted (black arrow). Bottom panel shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from embryonic High and Low LG-ABN offspring.

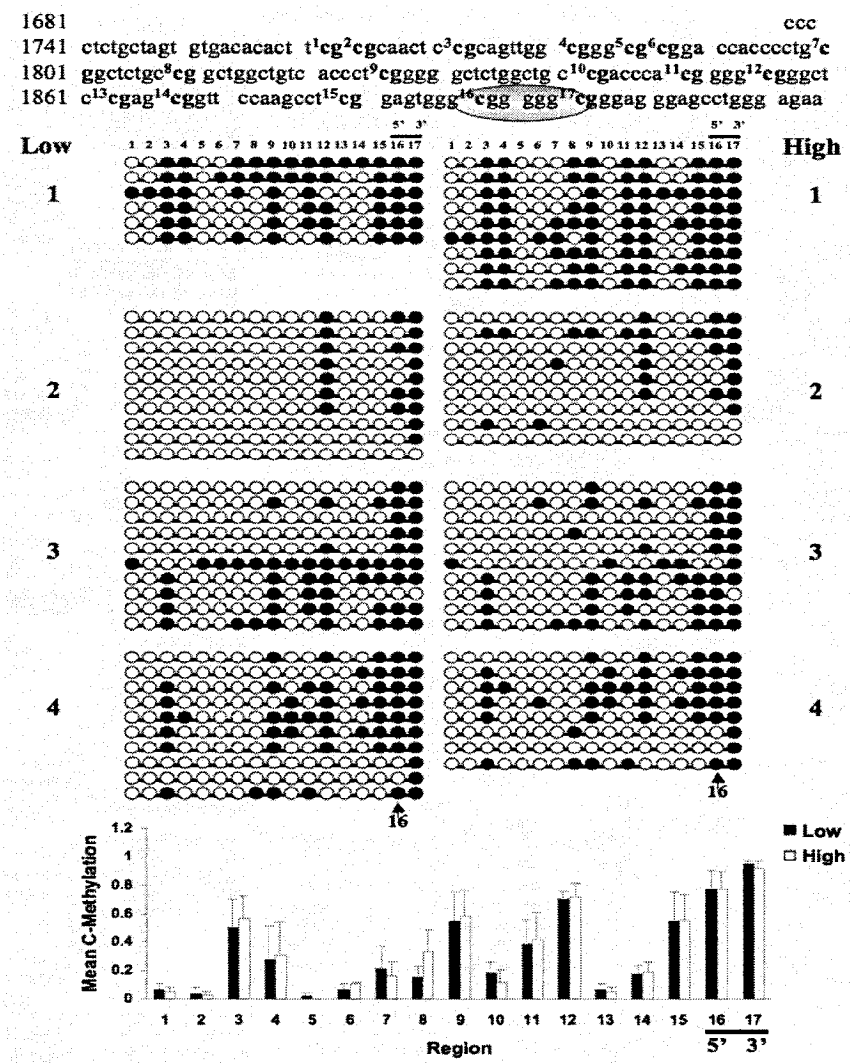

Figure 48: Methylation patterns of the exon 1_7 GR promoter in the hippocampi of 1-day-old (within one hour after birth) High and Low LG-ABN offspring (n=4 animals/group). Top panel shows a sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding region (encircled). Middle panel shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 GR promoter region analyzed by sodium bisulfite mapping (6-10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 GR promoter region are labelled 1-to-17, highlighting the two CpG dinucleotides [site 16 (5') and site 17 (3')] within the NGFI-A binding region (black line). Black circles indicate methylated cytosines and white circles indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5') is again highlighted (black arrow). Bottom panel shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from PND 1 High and Low LG offspring. The two-way ANOVA (Group x Region) revealed a highly significant effect of Region [$F=10.337$, $p<0.0001$].

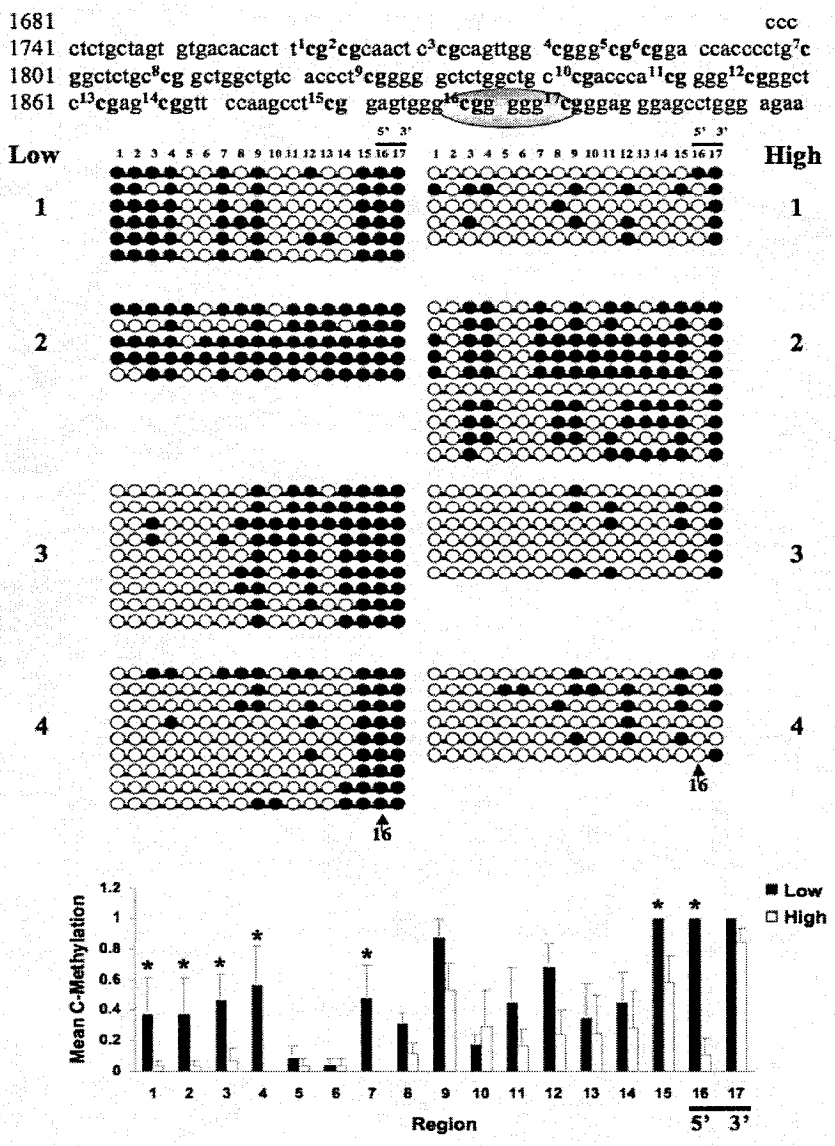

Figure 49: Methylation patterns of the exon 1_7 GR promoter in the hippocampi of 6-day-old (young pup) High and Low LG-ABN offspring (n=4 animals/group). Top panel shows a sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding region (encircled). Middle panel shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 GR promoter region analyzed by sodium bisulfite mapping (5-10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 GR promoter region are labelled 1-to-17, highlighting the two CpG dinucleotides [site 16 (5') and site 17 (3')] within the NGFI-A binding region (black line). Black circles indicate methylated cytosines and white circles indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5') is again highlighted (black arrow). Bottom panel shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region of the GR from PND 6 High and Low LG-ABN offspring [*$P < 0.05$; *$P < 0.0001$ for site 16 (5') lying within the NGFI-A binding region]. The two-way ANOVA (Group x Region) revealed a highly significant effect of both Group [F=32.569, p<0.0001] and Region [F=5.353, p<0.0001]. The Group x Region interaction effect was not significant [F=1.265, p=0.057].

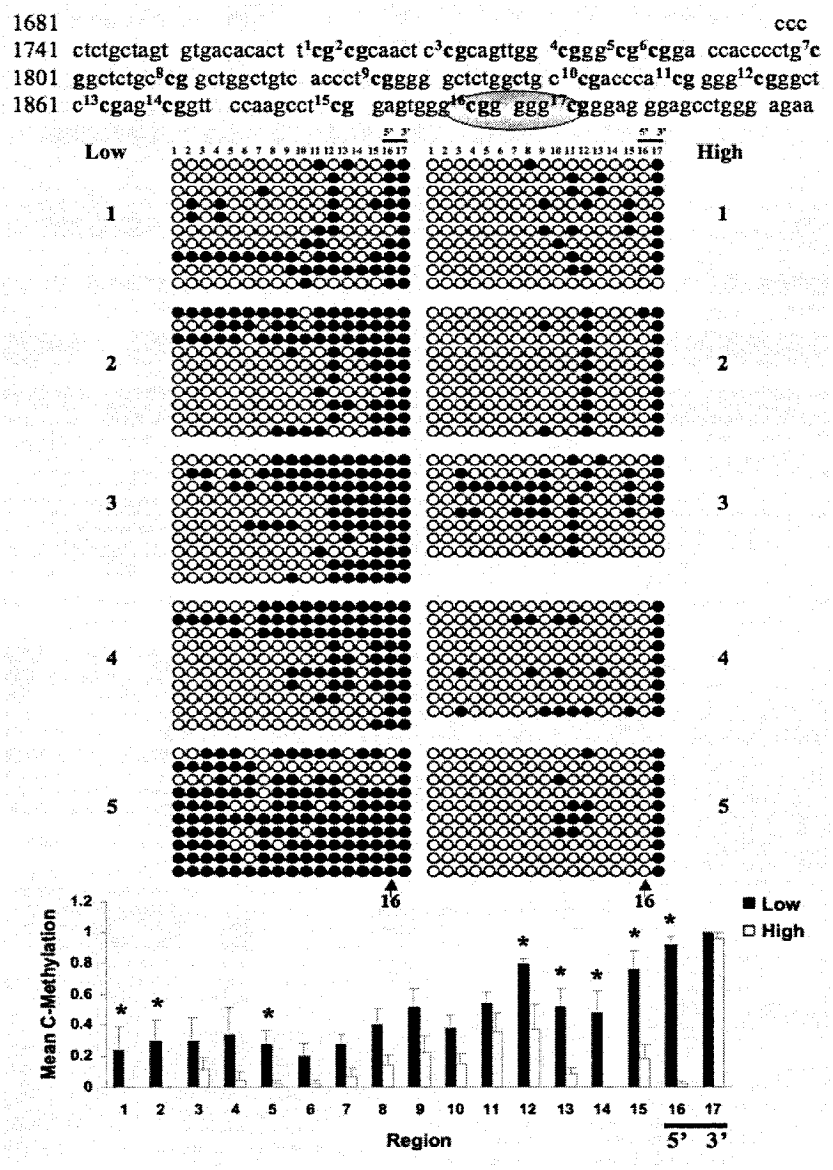

Figure 50: Methylation patterns of the exon 1_7 GR promoter in the hippocampi of 21-day-old (weaning age) High and Low LG-ABN offspring (n=4-5 animals/group). Top panel shows a sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding region (encircled). Middle panel shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 GR promoter region analyzed by sodium bisulfite mapping (6-10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 GR promoter region are labelled 1-to-17, highlighting the two CpG dinucleotides [site 16 (5') and site 17 (3')] within the NGFI-A binding region (black line). Black circles indicate methylated cytosines and white circles indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5') is again highlighted (black arrow). Bottom panel shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from PND 21 High and Low LG-ABN offspring (*$P < 0.05$). The two-way ANOVA (Group x Region) revealed a highly significant effect of both Group [$F=150.450$, $p<0.0001$] and Region [$F=12.474$, $p<0.0001$], as well as a significant Group x Region interaction effect [$F=4.223$, $p<0.0001$].

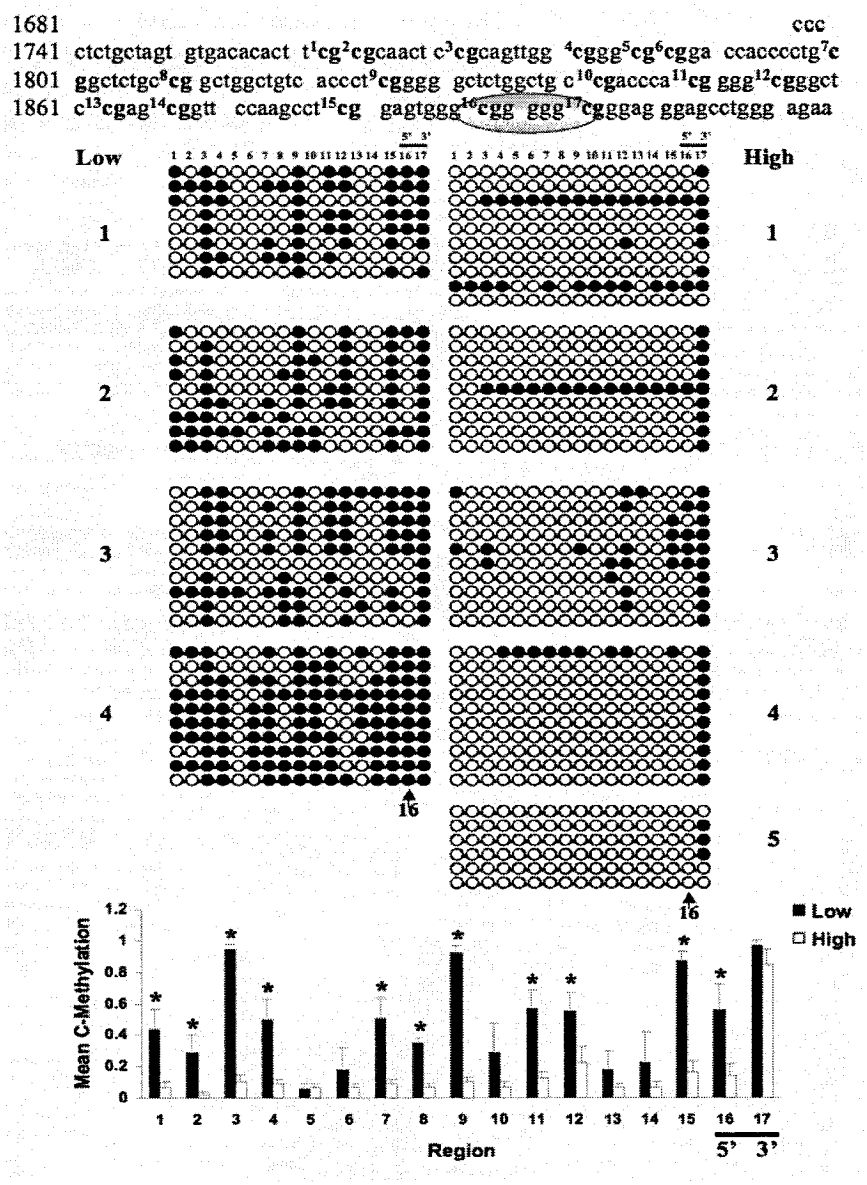

Figure 51: Methylation patterns of the exon 1_7 GR promoter in the hippocampi of 90-day-old (adult) High and Low LG-ABN offspring (n=5 animals/group). Top panel shows a sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding site (encircled). Middle panel shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 GR promoter region analyzed by sodium bisulfite mapping (8-10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 GR promoter region are labelled 1-to-17, highlighting the two CpG dinucleotides [site 16 (5') and site 17 (3')] within the NGFI-A binding region (black line). Black circles indicate methylated cytosines and white circles indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5') is again highlighted (black arrow). Bottom panel shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from PND 90 High and Low LG-ABN offspring [*$P < 0.05$; *$P < 0.0001$ for site 16 (5') lying within the NGFI-A binding region]. The two-way ANOVA (Group x Region) revealed a highly significant effect of Group [F=104.782, p<0.0001] and Region [F=11.443, p<0.0001], as well as a significant Group x Region interaction effect [F=2.321, p<0.01].

12.2. *Research Protocols Developed: Chemicals & Solutions*

12.2.1. Animal Tissue Exon 1_7 GR Promoter Gene Methylation Mapping

1) DNA extraction using DNeasy Tissue kit (250 rxns, Qiagen, cat #69506)
NB: If using fatty tissue, after adding 180 µl Buffer ATL and 20 µl Proteinase K, incubate samples at 55 °C overnight. Also, elute DNA from column with ***200 µl*** *10 mM Tris pH 8.5.*

2) Restriction enzyme digestion
i) add the following to an Eppendorph (1.7 ml):

	MM	
	extracted DNA (3-5 µg)	**10 µl**
(Fermentas, cat #ER0271)	EcoR1	4 µl
(Fermentas, cat #B12)	Buffer (with BSA)	20 µl
	ddH_2O	166 µl
		200 µl

ii) leave reaction tube in water bath (**3 h**, 37 °C)
iii) load **10 µl** of digestion reaction on an agarose gel (1.5 %)
NB: use tracking-dye (2 µl) & post-stain with Ethidium Bromide (10 µl/ddH_2O, ***15 min****)*

3) Phenol-chloroform DNA extraction
i) to the digested DNA (**190 µl**) add the following:

MM	
Phenol (0.5x)	100 µl
Chloroform (0.5x)	100 µl
NaCl (5 M, 0.1x)	20 µl
tRNA (10 mg/ml)	3 µl
	413 µl

ii) vortex & centrifuge (**15 min**, 13,200 rpm)
iii) resuspend **supernatant** (250 µl) in chloroform (250 µl, 0.5x)
iv) vortex & centrifuge (**15 min**, 13,200 rpm)
v) resuspend **supernatant** (250 µl) with twice volume (2 v/v) of ethanol (500 µl, 95 %)
vi) precipitate DNA(-80 °C, **30 min**)
vii) centrifuge (**15 min**, 13,200 rpm)
viii) wash **pellet** in ethanol (100 µl, 70 %) (NB: **do not vortex**, it will reduce DNA yield)
ix) centrifuge (**15 min**, 13,200 rpm)
x) resuspend **pellet** in ddH_2O (**54 µl**) [approx. 5 µg DNA]

4) Sodium bisulfite reaction
i) dissolve 7.49 g sodium bisulfite (Fisher Scientific cat# S654-500)] in ddH_2O (15 ml), and pH to 5 with 10N NaOH
ii) add hydroquinone (1 ml, 20 mM), and *qs* to 20 ml with ddH_2O
iii) transfer resuspended DNA (54 µl) into an Eppendorph (0.5 ml)
iv) add NaOH (6 µl, 3 M, final concentration of 0.3N)
v) incubate in water bath (37 °C,**15 min**)
vi) add sodium bisulfite solution [431 µl, sodium bisulfite (3.6 M)/hydroquinone (1 mM)]
vii) place samples on thermocycler: program (32) 'BIS':

step	temp	time
1	95 °C	2 min
2	55 °C	4 h
3	95 °C	2 min
4	55 °C	2 h
5	4 °C	1 min-24 h

viii) use QIAquick PCR purification kit (50 columns, Qiagen, cat #28104) (B1/P26)
*NB: elute DNA from column with **50 µl** 10 mM Tris pH 8.5 in to an Eppendorph (1.7 ml)*
ix) add NaOH (6 µl, 3M,final concentration of 0.3N NaOH)
x) incubate in water bath(**15 min**, 37° C)
xi) add NH_4OAc (26 µl, 10 M, final concentration 3M $NH_4OCH_3CO_2$) + tRNA (10 µl)
xii) add EtOH (300 µl, 95 %) & cool (**20 min**, -20 °C)
xii) centrifuge (**30 min**, 13,200 rpm, 4 °C)
xiii) lyophilise & resuspend pellet in ddH_2O (**100 µl**) [approx. 50 ng DNA/ 1 ml ddH_2O]

5) Sodium bisulfite treated DNA fragment amplification
i) design outside and nested primers. *NB: nested product = 200-400bp*
A) main rules:
1) primers must not contain any 5′-CpG-3′
2) outside & nested primers must NOT overlap, but can be right next to one another
3) design primers with annealing temp=56 °C (A=2 °C; T=2 °C; C=4°C; G=4 °C)
B) Forward primers:
1) write the nucleotide sequence (example: 5′-ACTGTACA-3′)
2) write the C-T conversion (example: 5′-ATTGTATA-3′)
3) calculate the annealing temperature (example: 2+2+2+4+2+2+2+2= 18 °C)
C) Reverse primers:
1) write the nucleotide sequence (example: 5′-TGTACATG-3′)
2) write the C-T conversion (example: 5′-TGTATATG -3′)
3) write the complementary sequence (example: 5′-CATATACA-3′)
4) calculate the annealing temperature (example: 4+2+2+2+2+2+4+2= 20 °C)
D) Order primers with a synthesis scale of 50 nM per primer
1) for primer stock solutions, calculate the amount of ddH_2O required to give a 500 µM solution, as follows: (OD X 180)/ No. of nucleotides = ? µl ddH_2O
2) for working solution (50 µM), dilute each primer stock solution (1/10) with ddH_20.
3) use **1 µl** of each primer working solution per 100 µl PCR reaction (0.5 mM)
ii) Preparing dNTP set
1) for working solution (10 mM), dilute 10 µl of each dNTP (100 mM) in 60 µl ddH_20
2) use **10 µl** of the dNTP working solution per 100 µl PCR reaction (0.2 mM)
iii) Preparing Taq polymerase
1) for working solution (1.25 U/µl) dilute Taq (5 U/µl) with ddH_20 (1/4)
2) use **1 µl** of the Taq working solution per 100 µl PCR reaction
iv) outside primer PCR; add the following to an Eppendorph (1.2 ml):

	MM	**1x**	**22x**
	Sodium bisulfite treated DNA	**1 µl**	
	ddH_2O	78 µl	
(MBI Fermentas, cat # EP0402)	10xPCR Buffer with $(NH_4)SO_4$	10 µl	220 µl

(MBI Fermentas, cat # R0181)	dNTP	2 µl	44 µl
	Forward primer	1 µl	22 µl
	Reverse primer	1 µl	22 µl
(MBI Fermentas, cat # EP0402)	$Mgcl_2$	6 µl	132 µl
(MBI Fermentas, cat # EP0402)	Taq pol [1/4 (v/v) with ddH_20]	1 µl	
		100 µl	

v) thermocycler **[3.5 h]**:

	step	temp	time
	1	95 °C	5 min
	2	95 °C	1 min
(temp=56 °C, grad=10)	3	51-61 °C	2.5 min
	4	72 °C	1 min
	5 go to step 2 for 34 cycles		
	6	72 °C	5 min
	7	4 °C	1 min-24 h

vi) nested primer PCR; add the following to a 1.2 ml PCR Eppendorph:

	MM	1x	22x
	Product from 1st PCR rxn	**5 µl**	
	ddH_2O	74 µl	
(MBI Fermentas, cat # EP0402)	10xPCR Buffer with $(NH_4)SO_4$	10 µl	220 µl
(MBI Fermentas, cat # R0181)	dNTP	2 µl	44 µl
	Forward primer	1 µl	22 µl
	Reverse primer	1 µl	22 µl
(MBI Fermentas, cat # EP0402)	$Mgcl_2$	6 µl	132 µl
(MBI Fermentas, cat # EP0402)	Taq pol [1/4 (v/v) with ddH_20]	1 µl	
		100 µl	

vii) use same thermocycler program as described above
viii) load **10 µl** of PCR product from nested primer reaction on an agarose gel (1.5 %)
*NB: use tracking-dye (2 µl) with only upper band & post-stain with Ethidium Bromide (10 µl/ddH_2O, **15 min**)*

6) use QIAquick PCR purification kit (50 columns, Qiagen, cat #28104)
*NB: elute DNA from column with **50 µl** 10 mM Tris pH 8.5 in to an Eppendorph (1.7 ml)*

7) Extraction of amplified NaBis DNA from agarose gel
i) load the entire recovered PCR product (**50 µl**) on to an agarose gel (1.5 %)
*NB: use tracking-dye (4 µl) with only upper band & post-stain with Ethidium Bromide (10 µl/ddH_2O, **15 min**)*
ii) cut out band corresponding to DNA fragment
iii) perform QIAquick II agarose gel extraction (150 rxns, Qiagen, cat #20021)
*NB: elute DNA with **20 µl** 10 mM Tris pH 8.5*

8) Plasmid ligation use Original TA cloning kit (Invitrogen, cat #K2000-40)
i) add the following to an Eppendorph (0.5 ml):

	MM	1x	10x
DNA		5 µl	50 µl

(Plasmid box #397)	PCR® 2.1 vector (4 kb)	2 µl	20 µl
	Buffer (10x)	1 µl	10 µl
	ddH_2O	1 µl	10 µl
	T4 ligase	1 µl	10 µl
		10 µl	**100 µl**

ii) thermocycler program: **ON (14 h)**, 15 °C

9) Plasmid transformation

i) make Laurier Broth (LB) by adding the following to a conical flask (1 L):

(Bio Shop, cat#TRP402)	Bio-tryptone	10 g
(Bio Shop, cat#YEX401)	Yeast extracts	5 g
	NaCl	10 g
	ddH_2O	*qs* **1 L**

ii) autoclave bottle (**20 min**, 121 °C) & allow cooling (**1 h**)

iii) make LB agar plates by adding the following to a 1 L flask:

(Bio Shop, cat#TRP402)	Bacto-tryptone	10 g
(Bio Shop, cat#YEX401)	Yeast extracts	5 g
	NaCl	10 g
	Agar	15 g
	ddH_2O	*qs* **1 L**

iv) autoclave bottle (**20 min**, 121 °C) & place bottle in water bath (**1 h**, 50 °C)

v) add ampicillin [**0.15 g**, 150 mg/ml, Fisher Biotech (25 gm), cat #BP1760-25] to LB

vi) pour plates (100 mm x 15 mm, Fisher brand, Cat#08-757-13) & store @ 4 °C

10) Transformation

i) add the following to an Eppendorph (1.7 ml):

	MM
Ligation product	10 µl
[TOPO 10 F' from cloning kit (Invitrogen, cat #K2000-40)]Competent cells	50 µl
	60 µl

ii) place on ice (**30 min**)

iii) heat shock cells in water bath (**1 min**, 45 °C) & place on ice (**1 min**)

iv) add 1 ml LB Amp^- (pre-warmed to 37 °C) & put on shaker (**30 min**, 200 rpm, 37 °C)

v) after approx. 30 min, plate x-gal (40µl, 40 mg/ml)

vi) centrifuge transformed bacteria grown in LB (**30 sec**, 8000 rpm)

vii) remove 800 µl of supernatant & resuspend **pellet** in remaining LB (approx. 200 µl)

viii) plate transformation (**100 µl**) & grow colonies [**ON (16 h)**, 37 °C]

11) Mini prep ('Jimmy Crack Corn' method)

i) choose white colonies with pipette tip, inoculate 2 ml LB Amp^+ [150 µg/ml (50 ml = 0.0075 g, 30 ml = 0.0045 g)] with clone, & grow colony [**ON (14 h)**, 37 °C] in 14 ml poly-propylene round-bottom Falcon tubes (Becton Dickinson Labware, cat# 4-2059-3)

***FOR NEXT STEP EITHER.....**

ii) transfer of the inoculated broth (2 x 750 µl) into an Eppendorph (1.7 ml)

iii) centrifuge (**2 min**, 6,000 rpm)
iv) make STET by adding the following to a conical flask (200 ml):

STET	
Sucrose	8.0 g
TritonX-100	0.5 ml
EDTA (0.5 M)	10.0 ml
Tris (1 M, pH 8.0)	0.5 ml
ddH_2O	*qs* **100 ml**

v) resuspend **pellet** in STET (300 µl), by raking tube (10x) across an Eppendorph rack

*.....**OR**,
i) remove pipette tips from Falcon tubes
ii) centrifuge Falcon tubes (4,000 rpm, 10 min, 4 C)
iii) remove supernatant from Falcon tubes using vacuum flask
iv) resuspend pellet in STET (300 µl)
v) transfer STET/pellet solution from Falcon tubes to a 1.7 ml Eppendorph.
NB: perform the following in sets of 5 and 10
vi) make fresh lysozyme by adding the following to an Eppendorph (1.7 ml):

	lysozyme (10 mg/ml) in Tris (25 mM, ph 8.0)	
(Sigma, cat# L7651)	Lysozyme	0.1 g [or, 10 µl of stock (10 mg/ ml)]
	Tris (2M, pH 7.6)	12.5 µl
	ddH_2O	1.0 ml

vii) add lysozyme (25 µl) to each rxn tube and mix by inversion (**3 sec**)
NB: Overdoing step (vi) results in increased amounts of RNA & chromosomal DNA
viii) immediately place samples into water bath(**45 sec**, 100 °C)
ix) centrifuge (**15 min**, 13,200 rpm)
x) Remove **pellet** (cellular debris/chromosomal DNA) with a toothpick (Touch Inc.)
Additional steps: a) add Chloroform (150 µl)
b) add Phenol (150 µl)
c) vortex & centrifuge (**5 min**, 13,200 rpm, 4 °C)
xi) add isopropanol (350 µl) to the **supernatant**
xii) vortex & cool (**5 min**, -20 °C; or **20 min**, RT)
xiii) centrifuge (**5 min**, 13,200 rpm, 4 °C)
xiv) wash **pellet** in ethanol (100 µl, 70 %)
xv) centrifuge (**5 min**, 13,200 rpm, 4 °C)
xvi) make TE (1x) by adding the following to a 50 ml tube:

TE (1x)	
Tris (1 M, pH 8.0)	500 µl
EDTA (0.5 M, pH 8.0)	100 µl
ddH_2O	*qs* **50 ml**

xvii) make fresh TE/RNase A by adding the following to a 1.7 ml Eppendorph tube:

	TE/RNase A	
(Roche, cat# 109169)	RNase A (stock:10 mg/ ml)	20 µl
	TE (1x)	1 ml
		1.020 ml

xviii) resuspend **pellet** in TE/RNase A (**50 µl**, 10 mg/ml)

NB: 10 µl/sequencing reaction (=5 rxns)

12) Restriction enzyme digestion (B#1/p158)
i) add the following to an Eppendorph (1.7 ml):

	MM	1x	44x
	Plasmid DNA	2 µl	88 µl
(Fermentas, cat #ER021)	EcoR1	1 µl	44 µl
(Fermentas, cat #B12)	Buffer (with BSA)	1 µl	44 µl
	ddH_2O	6 µl	264 µl
		10 µl	**440 µl**

ii) leave reaction tube in water bath (**3 h**, 37 °C)
iii) check digestion on an agarose gel (1.5 %)
*NB: use tracking-dye with only upper band & post-stain with Ethidium Bromide (10 µl/ddH_2O, **15 min**)*

13) T7Sequencing˙ Kit [100 rxns, Amersham Biosciences, Cat# 27-1682-01] (B#1/p160)
A) Denature dsDNA
i) add the following to an Eppendorph (1.7 ml):

dsDNA	10 µl
ddH_2O	22 µl
2 M NaOH	8 µl
	40 µl

ii) vortex, pulse with centrifuge, and incubate (**10 min**, RT)
iii) add the following:

Sodium acetate (3 M, pH 4.8)	7 µl
ddH_2O	4 µl
ethanol (95 %)	120 µl

iv) vortex, and precipitate DNA (**15 min**, -80 °C)
v) centrifuge (**15 min**, 13,200 rpm, 4 °C)
vi) pour-off supernatant and add ethanol (120 µl, 70 %)
vii) centrifuge (**10 min**, 13,200 rpm, 4 °C)
viii) remove supernatant (using a pipette set at 200 µl)
ix) redissolve pellet in ddH_2O (**10 µl**)
NB: samples can be stored at -20 °C

B) Annealing of primer: the point of NO return
i) add the following to the resuspended DNA template:

[Oligo box #143, (stock=500 µM) dilute 1/30]	Universal primer	2 µl
	Annealing buffer	2 µl
		14 µl

ii) vortex , pulse down with centrifuge, and incubate in water bath (**5 min**, 65 °C)
iii) incubate in water bath (**10 min**, 37 °C)
iv) incubate '**annealed template/primer**' (**5 min**, RT)
v) in preparation for each sample (*NB: 10 samples/gel*) to be sequenced, label four 1.7 ml Eppendorph tubes 'A', 'T', 'C', 'G' and add 2.5 µl of 'A' Mix-Short, 'T' Mix-Short, 'C' Mix-Short, 'G' Mix-Short '**sequencing mixes**' to each tube, respectively.
vi) make '**labelling reaction mix**' for 10 samples: **10x**

	T7 DNA polymerase	5 µl
	Dilution buffer	20 µl
	Labelling Mix A	36 µl
(Perkin-Elmer, cat#NEG-734H001MC)	dATP α ^{35}S [37.00 MBq (1.00 mCi)]	12 µl
		73 µl

vii) add 6 µl of the 'labelling reaction mix' to each 'annealed template/primer', pulse with centrifuge, and incubate at (5 min, RT)
viii) warm the four 'sequencing mixes'/sample in water bath (**at least 1 min**, 37 °C)
ix) add 4.5 µl of the 'labelling reaction mix & annealed primer' solution to each of the four sequencing mixes/sample, pulse with centrifuge and incubate in water bath (**5 min**, 37 °C)
x) add 5 µl of '**stop solution**' to each tube, pulse with centrifuge, and store (-20 °C)

14) Sequencing gel
i) make **10x TBE** (B#3/p1) by adding the following to a conical flask (1 L):

Tris base	121.0 g
Boric Acid	55.0 g
Disod EDTA	7.4 g
ddH_2O	*qs* **1 L**

ii) stir until dissolved
iii) make **denaturing gel (6 %)** by adding the following to a conical flask (100 ml):

Urea	50.0 g
Acrylamide /Bis (40 %, 19:1)	15.3 ml
TBE (10x)	10.0 ml
ddH_2O	*qs* **100 ml**

iv) stir until dissolved (**45 min**, RT)
v) polymerise denaturing gel by adding the following:

ammonium persulfate (APS)	0.050 g [or, 260 µl of stock (10 %)]
TEMED	130 µl

vi) pour denaturing gel & let stand (**1 h**, RT)
vii) place denaturing gel in chamber and add in TBE (1x)
viii) pre-run denaturing gel (**30 min**, 50-70 W, RT)
ix) boil sequencing samples (**2 min**)
x) load sequencing samples (3.5 µl/sample) & run PAGE (**3 h**, 50-70 W, RT)
NB: stop running PAGE when the 2nd dye-front band reaches the bottom of the gel
xi) transfer denaturing gel to Whatman paper and cover with syran wrap
xii) dry denaturing gel (**30 min**, 80 °C)
NB: remove syran wrap before exposing denaturing gel to film (approx. 24-48 h)

12.2.2. Animal Tissue Exon 1_7 GR Promoter Chromatin IP Assay

DAY 1
-prepare the following solutions:

1) **SDS Lysis buffer**:	5 ml SDS (10 %)	[SDS (1 %)]
	1 ml EDTA (0.5 M, pH 7.5)	[EDTA (1 mM)]
	2.5 ml Tris (1 M, pH 8)	[Tris (50 mM, pH 8)]
	qs 50 ml ddH_20	

2) **ChIP Dilution buffer**:	50 µl SDS (10 %)	[0.01 %]
(Sigma, Cat #T9284-500)	550 µl Triton X-100	[1.1 %]
	120 µl EDTA (5 M)	[1.2 mM]
	835 µl Tris (1 M, pH 8)	[16.7 mM, pH 8]
	1.67 ml NaCl (5 M)	[167 mM]
	qs 50 ml ddH_20	

3) **1xTE buffer**:	500 µl Tris (1 M, pH 8)	[10 mM, pH 8]
	100 µl EDTA (0.5 M)	[1 mM]
	qs 50 ml ddH_20	

4) **Low Salt Solution**:	500 µl SDS (10 %)	[1 %]
(Sigma, Cat #T9284-500)	500 µl Triton	[1 %]
	200 µl EDTA (0.5 M)	[2 mM]
	1 ml Tris (1 M, pH 8)	[20 mM, pH 8]
	1.5 ml NaCl (5 M)	[150 mM]
	qs 50 ml ddH_20	

5) **High Salt Solution**:	500 µl SDS (10 %)	[1 %]
(Sigma, Cat #T9284-500)	500 µl Triton	[1 %]
	200 µl EDTA (0.5 M)	[2 mM]
	1 ml Tris (1 M, pH 8)	[20 mM, pH 8]
	5 ml NaCl (5 M)	[500 mM]
	qs 50 ml ddH_20	

6) **LiCl Wash Buffer:**	0.3 g LiCl	[0.25 M]
(EM Science, Cat #B56009-70)	500 µl Nonidet P40 (NP_4O)	[1 %]
	0.5 g Deoxycholic acid	[1 %]
	100 µl EDTA (0.5 M)	[1 mM]
	500 µl Tris (1 M, pH 8)	[10 mM, pH 8]
	qs 50 ml ddH_20	

NB: filter each solution into fresh 50 ml tube using a 60 ml syringe (LUER-LOK™) connected to a 20 µm single use filter unit (Ministart®, Sartorius [Cat#16534]).

-prepare protein-G agarose/Herring sperm DNA slurry:

1) sonicate (40 %, 6x10 sec pulses) Herring sperm DNA (500 µl, 2 mg/ml) on ice
2) centrifuge (**4 min**, 3000 rpm, 4 °C) protein-G agarose (500 µl, Roche, Cat #1243233)
3) remove supernatant and resuspend ***pelleted*** protein-G agarose in the following:

	500 µl 1xTE
	100 µl sonicated Herring sperm DNA (2 µg/µl)
(Sigma, Cat CA7906-50G)	0.05 g Albumin from bovine serum [or, 10 µl BSA (50 ng/ml)] [0.5ng]

-prepare tissue for immuno-precipitation:

1) dilute a complete mini protease inhibitor tablet (Roche, Cat #1836-153) in SDS lysis buffer (10 ml) & cool (**10 min**, ice)
2) resuspend samples in SDS lysis/protease inhibitor buffer (200 µl)
3) sonicate (40 %, **6x10 sec pulses**) samples on ice
4) centrifuge (**10 min**, 13,000 rpm, 4 °C)
5) dilute (10-fold) ***supernatant*** in **ChIP dilution buffer** (1600 µl)
6) keep aliquots (100 µl) from each sample (label: '**INPUT**') and store (**O/N**, -80 °C)
7) add protein-G agarose/Herring sperm DNA slurry (80 µl) to remaining sample (pre-clear step)
8) place samples on nutator (**30 min**, 4 °C)
9) centrifuge (**5 min**, 3000 rpm, 4 °C)
10) divide sample into two: aliquot ***supernatant*** (900 µl) into two new Eppendorphs (1.5 ml)
11) add specific IgG or non-immune IgG primary antibody (10 µg) to each aliquot (900 µl)
12) place samples (with specific IgG or non-immune IgG) on nutator (**O/N**, 4 °C)

DAY 2

1) add protein-G agarose/Herring sperm DNA slurry (60 µl) to each sample
2) place samples (with specific IgG or non-immune IgG) on nutator (**1 h**, 4 °C)
3) centrifuge (**5 min**, 2500 rpm, 4 °C) [pellet = '**BOUND**']
4) keep supernatant from each sample (label: '**UNBOUND**') and store (-80 °C)

-perform washes of BOUND protein-DNA complexes:

1) resuspend pellet in **Low Salt buffer** (1 ml) and place on nutator (**5 min**, 4 °C)
2) centrifuge (**5 min**, 2500 rpm, 4 °C) and discard ***supernatant***
3) resuspend pellet in **High Salt buffer** (1 ml) and place on nutator (**5 min**, 4 °C)
4) centrifuge (**5 min**, 2500 rpm, 4 °C) and discard ***supernatant***
5) resuspend pellet in **LiCl buffer** (1 ml) and place on nutator (**5 min**, 4 °C)
6) centrifuge (**5 min**, 2500 rpm, 4 °C) and discard ***supernatant***
7) resuspend pellet in **1xTE** (1 ml) and place on nutator (**5 min**, 4 °C)
8) centrifuge (**5 min**, 2500 rpm, 4 °C) and discard ***supernatant***
9) repeat steps 7-8 (5x)

-elution of chromatin immuno-precipitate:

1) prepare **Elution buffer**:

   5 ml SDS (10 %)
   0.42 g sodium bicarbonate
   qs 50 ml ddH_20

2) resuspend (by vortexing) each pellet in **Elution buffer** (250 µl)
3) place samples on nutator (**15 min**, RT)
4) centrifuge (**5 min**, 2500 rpm, RT)
5) aliquot ***supernatant*** (250 µl) into a new Eppendorph (1.5 ml)
10) repeat steps 2-4 and ***pool supernatant*** (250 µl + 250 µl = 500 µl) and store (**O/N**, -80 °C)

DAY 3

1) dilute all '**INPUT**' samples with **Elution buffer** (400 µl)

-uncross-link the protein-DNA complex from all samples (including 'INPUT' samples):

1) to each sample add NaCl (20 µl, 5 M) and incubate (**4 h**, 65 °C)
2) add the following to each sample:

10 µl EDTA (0.5 M, pH 7.5)
20 µl TrisHCl (1 M, pH 6.5)
2 µl Proteinase K (stock = 10 mg/ml)

3) incubate samples (**1 h**, 45 °C)
4) add phenol (275 µl) and chloroform (275 µl)
5) vortex and centrifuge (**5 min**, 13,200 rpm, 4 °C)
6) ***transfer aqueous phase*** to an Eppendorph (1.5 ml) with Ethanol (1 ml, 95 %) and tRNA (5 µl)
7) cool samples (**30 min**, -80 °C)
8) centrifuge (**15 min**, 13,200 rpm, 4 °C) and discard ***supernatant***
9) wash pellet with Ethanol (100 µl, 70 %)
10) centrifuge (**5 min**, 13,200 rpm, 4 °C) and discard ***supernatant***
11) resuspend DNA ***pellet*** in 1xTE (100 µl)

-DNA fragment amplification:

1) add the following to an Eppendorph (1.2 ml) ***(B#4/P78)***:

	MM	**1x**	**13x**	**26x**
[739]	Forward primer (50 µM)	1 µl	13 µl	26 µl
[740]	Reverse primer (50 µM)	1 µl	13 µl	26 µl
	ddH_2O	16 µl	208 µl	416 µl
	Buffer D (Epicentre®, Cat *#FSP995D)*	25 µl	325 µl	650 µl
		43 µl	**559 µl**	**1118 µl**

2) add **MM** (43 µl) to each reaction tube containing ***immunoprecipitated DNA*** **(6 µl)**
3) add Fail Safe™ enzyme [1 µl (¼ dilution), Epicentre®, Cat *#FSE51100*] each reaction tube
4) thermocycler program [**2.5 h**]:

step	temp	time
1	95 °C	5 min
2	95 °C	1 min
3	56 °C	1 min
4	72 °C	1 min
5 go to step 2 for 34 cycles		
6	72 °C	10 min
7	4 °C	1 min-24 h

5) load **10 µl** of PCR product [with only upper-band tracking-dye (2 µl)] on an agarose gel (2 %)
6) post-stain with Ethidium Bromide (10 µl/ddH_2O, **15 min**)

-Southern blot analysis:

1) prepare **NaOH (0.4 M)**:

[1 M = 40 g/l, 0.4 M = 16 g/l] 20 ml NaOH (20 N)
[8 g NaOH/ 500 ml ddH_2O] **qs 500 ml ddH_2O**

2) perform **NaOH (0.4 M)** mediated transfer of DNA from gel to **Hybond N^+** nylon transfer membrane (**O/N**, RT, Amersham biosciences, Cat #RPN303B)

3) wash membrane in ddH_2O (5 min, RT) and dry membrane (RT) compressed between filter paper

DAY 4

-oligonucleotide end-labelling

1) make **oligonucleotide end-labelling mix** by adding the following to an Eppendorph (1.2 ml):

(MBI Fermentas, Cat #EK0031)	1 µl T4 PNK enzyme (10 u/µl, 500 u)
	5 µl PNK 10xbuffer [(A)-Forward]
	1 µl 1/10 dilution of oligonucleotide
[250 µCi (9.25 MBq), Perkin-Elmer, Cat #BLU502Z]	5 µl γ-^{32}P ATP (10 mCi/ml)
	38 µl ddH_2O
	50 µl

2) incubate **oligonucleotide end-labelling mix** (**1 h**, 37 °C)

-prehybridize membrane:

1) Denhardt's solution (store @ -20 °C):

20g	Ficoll-400
20g	Polyvinyl pyrrolidone (PVP)
20g	Albumin from bovine serum
qs 1L ddH_20	

2) make **pre-hybridizing buffer** by adding the following to a 15 ml tube:

3 ml 20xSSC (6x)
1 ml 10% SDS (1%)
0.5 ml 100xDenhardt's (5x)
0.05 g tetrasodium pyrophosphate (0.05 %)
qs 10 ml ddH_20

3) incubate (**20 min**, 65 °C)

4) add herring sperm (100 µl)

5) place hybridization tube with **pre-hybridization buffer**/membrane in hyb-chamber (**2 h**, 42 °C)

-TCA precipitation:

1) make **TCA solution** by adding the following to a 15 ml tube:

1.5 ml TCA (10 %)
30 µl herring sperm
2 µl labelled probe

2) incubate **TCA solution** on ice (**10 min**, 4 °C)

3) pour solution over filter

4) wash filer twice with TCA (10 %, 5 ml)

5) pass filter (5 ml scintillation fluid) through scintillation counter (range = 1 million counts)

6) add **oligonucleotide end-labelling mix** to membrane & re-place to hyb-chamber (**O/N**, 42 °C)

DAY 5

-perform membrane washes:

1) make **membrane wash buffer**:

300 ml 20xSSC (500 ml 6xSSC)
100 ml 10% SDS (100 ml 1% SDS)
0.5 g tetrasodium pyrophosphate (0.05 %)
qs 1 L dd$H_2$0

2) incubate **membrane wash buffer** (**20 min**, 65 °C)
3) wash membrane 1x (**5 min each**, RT)
4) wash membrane 2x (**10 min each**, RT)
5) wash membrane 4x (**10 min each**, 55 °C)
-expose membrane to film and develop:
-strip membrane:
1) wash membrane in metal tray containing 0.5 % SDS (boil, 20 min or until Geiger < 10 hms)

Chapter 13
References

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.
Adams, R. L., McKay, E. L., Craig, L. M., and Burdon, R. H. (1979). Mouse DNA methylase: methylation of native DNA. Biochim Biophys Acta *561*, 345-357.
Adolphs, R., Tranel, D., Damasio, H., and Damasio, A. R. (1995). Fear and the human amygdala. J Neurosci *15*, 5879-5891.
Aggleton, J. P. (1993). The contribution of the amygdala to normal and abnormal emotional states. Trends Neurosci *16*, 328-333.
Agrawal, A. A. (2001). Phenotypic plasticity in the interactions and evolution of species. Science *294*, 321-326.
Aguilera, G., Kiss, A., and Sunar-Akbasak, B. (1995). Hyperreninemic hypoaldosteronism after chronic stress in the rat. J Clin Invest *96*, 1512-1519.
Ainsworth, A. J., and Brown, J. E. (1991). Detection of in ovo derived idiotypic antibodies. I. A model for maternal-neonatal idiotype network studies. J Immunol *147*, 910-914.
Akner, G., Wikstrom, A. C., Mossberg, K., Sundqvist, K. G., and Gustafsson, J. A. (1994). Morphometric studies of the localization of the glucocorticoid receptor in mammalian cells and of glucocorticoid hormone-induced effects. J Histochem Cytochem *42*, 645-657.
Alberts, J. R., and Leimbach, M. P. (1980). The first foray: maternal influences in nest egression in the weanling rat. Dev Psychobiol *13*, 417-429.
Allen, R. T., Hunter, W. J., 3rd, and Agrawal, D. K. (1997). Morphological and biochemical characterization and analysis of apoptosis. J Pharmacol Toxicol Methods *37*, 215-228.
Altman, J., and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol *124*, 319-335.
Amaral, D. G., and Witter, M. P. (1989). The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience *31*, 571-591.
Amiragova, M. G. (1985). Neurophysiological analysis of the development of endocrine and hypertensive reactions in prolonged emotional stress. Brain Res *344*, 303-315.
Ammerman, R. T. (1991). The role of the child in physical abuse: a reappraisal. Violence Vict *6*, 87-101.
Anand, K. J., and Scalzo, F. M. (2000). Can adverse neonatal experiences alter brain development and subsequent behavior? Biol Neonate *77*, 69-82.
Anisman, H., Zaharia, M. D., Meaney, M. J., and Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? Int J Dev Neurosci *16*,

149-164.
Antequera, F., and Bird, A. (1993). CpG islands. Exs *64*, 169-185.
Antonsson, B., and Martinou, J. C. (2000). The Bcl-2 protein family. Exp Cell Res *256*, 50-57.
Apfel, S. C., Wright, D. E., Wiideman, A. M., Dormia, C., Snider, W. D., and Kessler, J. A. (1996). Nerve growth factor regulates the expression of brain-derived neurotrophic factor mRNA in the peripheral nervous system. Mol Cell Neurosci *7*, 134-142.
Arlt, W., and Stewart, P. M. (2005). Adrenal corticosteroid biosynthesis, metabolism, and action. Endocrinol Metab Clin North Am *34*, 293-313, viii.
Aronsson, M., Fuxe, K., Dong, Y., Agnati, L. F., Okret, S., and Gustafsson, J. A. (1988). Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci U S A *85*, 9331-9335.
Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science *237*, 268-275.
Ashkenazi, A., and Dixit, V. M. (1998). Death receptors: signaling and modulation. Science *281*, 1305-1308.
Ayer, D. E. (1999). Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends Cell Biol *9*, 193-198.
Bachman, K. E., Park, B. H., Rhee, I., Rajagopalan, H., Herman, J. G., Baylin, S. B., Kinzler, K. W., and Vogelstein, B. (2003). Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell *3*, 89-95.
Ballard, P. L. (1979). Delivery and transport of glucocorticoids to target cells. Monogr Endocrinol *12*, 25-48.
Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. (1996). Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev *17*, 245-261.
Banker, G. A., and Cowan, W. M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res *126*, 397-342.
Barden, N., Stec, I. S., Montkowski, A., Holsboer, F., and Reul, J. M. (1997). Endocrine profile and neuroendocrine challenge tests in transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuroendocrinology *66*, 212-220.
Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Colch) *94*, 557-572.
Barth, M., Schilling, L., and Schmiedek, P. (2000). Time course of apoptotic cell death after experimental neurotrauma. Acta Neurochir Suppl *76*, 121-124.
Bauer, A., Tronche, F., Wessely, O., Kellendonk, C., Reichardt, H. M., Steinlein, P., Schutz, G., and Beug, H. (1999). The glucocorticoid receptor is required for stress erythropoiesis. Genes Dev *13*, 2996-3002.
Bauer, M. E. (2005). Stress, glucocorticoids and ageing of the immune system. Stress *8*, 69-83.
Beato, M. (1987). Induction of transcription by steroid hormones. Biochim Biophys Acta *910*, 95-102.
Beato, M. (1991). Transcriptional control by nuclear receptors. Faseb J *5*, 2044-2051.
Beato, M., Chalepakis, G., Schauer, M., and Slater, E. P. (1989). DNA regulatory elements for steroid hormones. J Steroid Biochem *32*, 737-747.

Beato, M., Herrlich, P., and Schutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.
Beaulieu, S., Di Paolo, T., and Barden, N. (1986). Control of ACTH secretion by the central nucleus of the amygdala: implication of the serotoninergic system and its relevance to the glucocorticoid delayed negative feedback mechanism. Neuroendocrinology *44*, 247-254.
Beaulieu, S., Di Paolo, T., Cote, J., and Barden, N. (1987). Participation of the central amygdaloid nucleus in the response of adrenocorticotropin secretion to immobilization stress: opposing roles of the noradrenergic and dopaminergic systems. Neuroendocrinology *45*, 37-46.
Beck, C. D., and Rankin, C. H. (1995). Heat shock disrupts long-term memory consolidation in Caenorhabditis elegans. Learn Mem *2*, 161-177.
Beckmann, A. M., and Wilce, P. A. (1997). Egr transcription factors in the nervous system. Neurochem Int *31*, 477-510; discussion 517-476.
Beerli, R. R., and Barbas, C. F., 3rd (2002). Engineering polydactyl zinc-finger transcription factors. Nat Biotechnol *20*, 135-141.
Bereiter, D. A., and Gann, D. S. (1986). Caudolateral areas of medulla-mediating release of ACTH in cats. Am J Physiol *251*, R934-940.
Berg, J. M. (1989). DNA binding specificity of steroid receptors. Cell *57*, 1065-1068.
Bertini, R., Bianchi, M., Villa, P., and Ghezzi, P. (1988). Depression of liver drug metabolism and increase in plasma fibrinogen by interleukin 1 and tumor necrosis factor: a comparison with lymphotoxin and interferon. Int J Immunopharmacol *10*, 525-530.
Bestor, T. (1988). Structure of mammalian DNA methyltransferase as deduced from the inferred amino acid sequence and direct studies of the protein. Biochem Soc Trans *16*, 944-947.
Bestor, T. H. (1992). Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. Embo J *11*, 2611-2617.
Bestor, T. H., and Verdine, G. L. (1994). DNA methyltransferases. Curr Opin Cell Biol *6*, 380-389.
Bhatnagar, S., Dallman, M. F., Roderick, R. E., Basbaum, A. I., and Taylor, B. K. (1998). The effects of prior chronic stress on cardiovascular responses to acute restraint and formalin injection. Brain Res *797*, 313-320.
Bhatnagar, S., and Meaney, M. J. (1995). Hypothalamic-pituitary-adrenal function in chronic intermittently cold-stressed neonatally handled and non handled rats. J Neuroendocrinol *7*, 97-108.
Bhattacharya, S. K., Ramchandani, S., Cervoni, N., and Szyf, M. (1999). A mammalian protein with specific demethylase activity for mCpG DNA. Nature *397*, 579-583.
Bird, A. (2001). Molecular biology. Methylation talk between histones and DNA. Science *294*, 2113-2115.
Bird, A. (2003). Il2 transcription unleashed by active DNA demethylation. Nat Immunol *4*, 208-209.
Bird, A. P. (1996). The relationship of DNA methylation to cancer. Cancer Surv *28*, 87-101.
Bird, A. P., and Wolffe, A. P. (1999). Methylation-induced repression--belts, braces, and chromatin. Cell *99*, 451-454.
Blair, D. M., and Halperin, H. R. (1996). Hand-held, dynamic indentation system for measuring myocardial transverse stiffness. Biomed Instrum Technol *30*, 517-525.

Blancafort, P., Segal, D. J., and Barbas, C. F., 3rd (2004). Designing transcription factor architectures for drug discovery. Mol Pharmacol *66*, 1361-1371.
Bliss, T. V., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol *232*, 331-356.
Bonaz, B., and Tache, Y. (1994). Induction of Fos immunoreactivity in the rat brain after cold-restraint induced gastric lesions and fecal excretion. Brain Res *652*, 56-64.
Bourguet, W., Ruff, M., Bonnier, D., Granger, F., Boeglin, M., Chambon, P., Moras, D., and Gronemeyer, H. (1995). Purification, functional characterization, and crystallization of the ligand binding domain of the retinoid X receptor. Protein Expr Purif *6*, 604-608.
Boyle, M. P., Brewer, J. A., Funatsu, M., Wozniak, D. F., Tsien, J. Z., Izumi, Y., and Muglia, L. J. (2005). Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc Natl Acad Sci U S A *102*, 473-478.
Branchi, I., Santucci, D., and Alleva, E. (2001). Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav Brain Res *125*, 49-56.
Brandeis, M., Ariel, M., and Cedar, H. (1993). Dynamics of DNA methylation during development. Bioessays *15*, 709-713.
Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994). Sp1 elements protect a CpG island from de novo methylation. Nature *371*, 435-438.
Bredy, T. W., Grant, R. J., Champagne, D. L., and Meaney, M. J. (2003a). Maternal care influences neuronal survival in the hippocampus of the rat. Eur J Neurosci *18*, 2903-2909.
Bredy, T. W., Humpartzoomian, R. A., Cain, D. P., and Meaney, M. J. (2003b). Partial reversal of the effect of maternal care on cognitive function through environmental enrichment. Neuroscience *118*, 571-576.
Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1989). Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. J Biol Chem *264*, 4992-4997.
Brindley, D. N., and Rolland, Y. (1989). Possible connections between stress, diabetes, obesity, hypertension and altered lipoprotein metabolism that may result in atherosclerosis. Clin Sci (Colch) *77*, 453-461.
Brinton, R. D., Yamazaki, R., Gonzalez, C. M., O'Neill, K., and Schreiber, S. S. (1998). Vasopressin-induction of the immediate early gene, NGFI-A, in cultured hippocampal glial cells. Brain Res Mol Brain Res *57*, 73-85.
Brouette-Lahlou, I., Godinot, F., and Vernet-Maury, E. (1999). The mother rat's vomeronasal organ is involved in detection of dodecyl propionate, the pup's preputial gland pheromone. Physiol Behav *66*, 427-436.
Brown, M. S., Kovanen, P. T., and Goldstein, J. L. (1979). Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. Recent Prog Horm Res *35*, 215-257.
Bruniquel, D., and Schwartz, R. H. (2003). Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol *4*, 235-240.
Bucan, M., and Abel, T. (2002). The mouse: genetics meets behaviour. Nat Rev Genet *3*, 114-123.
Burek, M. J., and Oppenheim, R. W. (1996). Programmed cell death in the developing

nervous system. Brain Pathol *6*, 427-446.
Burton, R. M., Harding, G. B., Rust, N., and Westphal, U. (1971). Steroid-protein interactions. 23. Nonidentity of cortisol-binding globulin and progesterone-binding globulin in guinea pig serum. Steroids *17*, 1-16.
Butler, S. R., and Schanberg, S. M. (1977). Effect of maternal deprivation on polyamine metabolism in preweanling rat brain and heart. Life Sci *21*, 877-884.
Butler, S. R., Suskind, M. R., and Schanberg, S. M. (1978). Maternal behavior as a regulator of polyamine biosynthesis in brain and heart of the developing rat pup. Science *199*, 445-447.
Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P. M., and Meaney, M. J. (1998). Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc Natl Acad Sci U S A *95*, 5335-5340.
Calhoun, J. B. (1962). The ecology and sociology of the Norway Rat (Bethesda, MD, USA: Public Health Service).
Campeau, S., Hayward, M. D., Hope, B. T., Rosen, J. B., Nestler, E. J., and Davis, M. (1991). Induction of the c-fos proto-oncogene in rat amygdala during unconditioned and conditioned fear. Brain Res *565*, 349-352.
Cannon, W. B. (1932). The wisdom of the body (New York: Norton).
Cantoni, G. L. (1975). Biological methylation: selected aspects. Annu Rev Biochem *44*, 435-451.
Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990). Steroid receptor family: structure and functions. Endocr Rev *11*, 201-220.
Caruncho, H. J., Dopeso-Reyes, I. G., Loza, M. I., and Rodriguez, M. A. (2004). A GABA, reelin, and the neurodevelopmental hypothesis of schizophrenia. Crit Rev Neurobiol *16*, 25-32.
Carvin, C. D., Parr, R. D., and Kladde, M. P. (2003). Site-selective in vivo targeting of cytosine-5 DNA methylation by zinc-finger proteins. Nucleic Acids Res *31*, 6493-6501.
Catalano, R. D., Stuve, L., and Ramachandran, J. (1986). Characterization of corticotropin receptors in human adrenocortical cells. J Clin Endocrinol Metab *62*, 300-304.
Cattaneo, E., and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature *347*, 762-765.
Cervoni, N., Detich, N., Seo, S. B., Chakravarti, D., and Szyf, M. (2002). The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem *277*, 25026-25031.
Cervoni, N., and Szyf, M. (2001). Demethylase activity is directed by histone acetylation. J Biol Chem *27*, 27.
Chalepakis, G., Schauer, M., Cao, X. A., and Beato, M. (1990). Efficient binding of glucocorticoid receptor to its responsive element requires a dimer and DNA flanking sequences. DNA Cell Biol *9*, 355-368.
Champagne, F. A., Chretien, P., Stevenson, C. W., Zhang, T. Y., Gratton, A., and Meaney, M. J. (2004). Variations in nucleus accumbens dopamine associated with individual differences in maternal behavior in the rat. J Neurosci *24*, 4113-4123.
Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol Behav *79*, 359-371.
Chao, H. M., Choo, P. H., and McEwen, B. S. (1989). Glucocorticoid and mineralocorti-

coid receptor mRNA expression in rat brain. Neuroendocrinology *50*, 365-371.
Cheal, M. L., and Sprott, R. L. (1971). Social olfaction: a review of the role of olfaction in a variety of animal behaviors. Psychol Rep *29*, 195-243.
Choi, D. W. (1995). Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci *18*, 58-60.
Cierpial, M. A., Murphy, C. A., and McCarty, R. (1990). Maternal behavior of spontaneously hypertensive and Wistar-Kyoto normotensive rats: effects of reciprocal cross-fostering of litters. Behav Neural Biol *54*, 90-96.
Cintra, A., Bhatnagar, M., Chadi, G., Tinner, B., Lindberg, J., Gustafsson, J. A., Agnati, L. F., and Fuxe, K. (1994a). Glial and neuronal glucocorticoid receptor immunoreactive cell populations in developing, adult, and aging brain. Ann N Y Acad Sci *746*, 42-61; discussion 61-43.
Cintra, A., Zoli, M., Rosen, L., Agnati, L. F., Okret, S., Wikstrom, A. C., Gustaffsson, J. A., and Fuxe, K. (1994b). Mapping and computer assisted morphometry and microdensitometry of glucocorticoid receptor immunoreactive neurons and glial cells in the rat central nervous system. Neuroscience *62*, 843-897.
Cirulli, F., Berry, A., and Alleva, E. (2003). Early disruption of the mother-infant relationship: effects on brain plasticity and implications for psychopathology. Neurosci Biobehav Rev *27*, 73-82.
Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994). High sensitivity mapping of methylated cytosines. Nucleic Acids Res *22*, 2990-2997.
Cole, T. J., Blendy, J. A., Monaghan, A. P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., and Schutz, G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev *9*, 1608-1621.
Cole, T. J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Schutz, G., and Ruppert, S. (1992). The mouse CREB (cAMP responsive element binding protein) gene: structure, promoter analysis, and chromosomal localization. Genomics *13*, 974-982.
Comb, M., and Goodman, H. M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res *18*, 3975-3982.
Cooney, C. A. (1993). Are somatic cells inherently deficient in methylation metabolism? A proposed mechanism for DNA methylation loss, senescence and aging. Growth Dev Aging *57*, 261-273.
Cooney, C. A., Dave, A. A., and Wolff, G. L. (2002). Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr *132*, 2393S-2400S.
Cowan, W. M., Fawcett, J. W., O'Leary, D. D., and Stanfield, B. B. (1984). Regressive events in neurogenesis. Science *225*, 1258-1265.
Crabbe, J. C., Wahlsten, D., and Dudek, B. C. (1999). Genetics of mouse behavior: interactions with laboratory environment. Science *284*, 1670-1672.
Cramer, C. P., Thiels, E., and Alberts, J. R. (1990). Weaning in rats: I. Maternal behavior. Dev Psychobiol *23*, 479-493.
Crane-Robinson, C., Myers, F. A., Hebbes, T. R., Clayton, A. L., and Thorne, A. W. (1999). Chromatin immunoprecipitation assays in acetylation mapping of higher eukaryotes. Methods Enzymol *304*, 533-547.
Cratty, M. S., Ward, H. E., Johnson, E. A., Azzaro, A. J., and Birkle, D. L. (1995). Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. Brain Res *675*, 297-302.

Crawley, J. N. (1985). Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev *9*, 37-44.
Crino, P., Khodakhah, K., Becker, K., Ginsberg, S., Hemby, S., and Eberwine, J. (1998). Presence and phosphorylation of transcription factors in developing dendrites. Proc Natl Acad Sci U S A *95*, 2313-2318.
Crooke, S. T. (2004). Progress in antisense technology. Annu Rev Med *55*, 61-95.
Crosby, S. D., Puetz, J. J., Simburger, K. S., Fahrner, T. J., and Milbrandt, J. (1991). The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol Cell Biol *11*, 3835-3841.
Crosio, C., Heitz, E., Allis, C. D., Borrelli, E., and Sassone-Corsi, P. (2003). Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons. J Cell Sci *116*, 4905-4914.
Croskerry, P. G., Smith, G. K., and Leon, M. (1978). Thermoregulation and the maternal behaviour of the rat. Nature *273*, 299-300.
Dahlman-Wright, K., Wright, A., Gustafsson, J. A., and Carlstedt-Duke, J. (1991). Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem *266*, 3107-3112.
Dallman, M. F., Akana, S. F., Jacobson, L., Levin, N., Cascio, C. S., and Shinsako, J. (1987). Characterization of corticosterone feedback regulation of ACTH secretion. Ann N Y Acad Sci *512*, 402-414.
Daly, M. (1973). Early stimulation of rodents: a critical review of present interpretations. Br J Psychol *64*, 435-460.
Danielsen, M., Northrop, J. P., Jonklaas, J., and Ringold, G. M. (1987). Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. Mol Endocrinol *1*, 816-822.
Danielsen, M., Northrop, J. P., and Ringold, G. M. (1986). The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. Embo J *5*, 2513-2522.
Davie, J. R., and Chadee, D. N. (1998). Regulation and regulatory parameters of histone modifications. J Cell Biochem Suppl *30-31*, 203-213.
Davis, M. (1994). The role of the amygdala in emotional learning. Int Rev Neurobiol *36*, 225-266.
De Kloet, E. R., and Reul, J. M. (1987). Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology *12*, 83-105.
De Kloet, E. R., Veldhuis, H. D., Wagenaars, J. L., and Bergink, E. W. (1984). Relative binding affinity of steroids for the corticosterone receptor system in rat hippocampus. J Steroid Biochem *21*, 173-178.
De Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., and Joels, M. (1998). Brain corticosteroid receptor balance in health and disease. Endocr Rev *19*, 269-301.
Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, E. M., Jr., Snider, W. D., and Korsmeyer, S. J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron *17*, 401-411.
Denenberg, V. H. (1999). Commentary: is maternal stimulation the mediator of the handling effect in infancy? Dev Psychobiol *34*, 1-3.

Deshmukh, M., and Johnson, E. M., Jr. (1998). Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c [see comments]. Neuron *21*, 695-705.
Detich, N., Hamm, S., Just, G., Knox, J. D., and Szyf, M. (2003). The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine. J Biol Chem *278*, 20812-20820.
Diamond, D. M., Bennett, M. C., Fleshner, M., and Rose, G. M. (1992). Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. Hippocampus *2*, 421-430.
Dingwall, C., and Laskey, R. A. (1991). Nuclear targeting sequences--a consensus? Trends Biochem Sci *16*, 478-481.
Dirnagl, U., Iadecola, C., and Moskowitz, M. A. (1999). Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci *22*, 391-397.
Dong, E., Caruncho, H., Liu, W. S., Smalheiser, N. R., Grayson, D. R., Costa, E., and Guidotti, A. (2003). A reelin-integrin receptor interaction regulates Arc mRNA translation in synaptoneurosomes. Proc Natl Acad Sci U S A *100*, 5479-5484.
Drouin, J., Sun, Y. L., Tremblay, S., Lavender, P., Schmidt, T. J., de Lean, A., and Nemer, M. (1992). Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor. Mol Endocrinol *6*, 1299-1309.
Duncan, G. E., Knapp, D. J., Johnson, K. B., and Breese, G. R. (1996). Functional classification of antidepressants based on antagonism of swim stress-induced fos-like immunoreactivity. J Pharmacol Exp Ther *277*, 1076-1089.
Durand, P., Fortin, L. J., Lussier-Cacan, S., Davignon, J., and Blache, D. (1996). Hyperhomocysteinemia induced by folic acid deficiency and methionine load--applications of a modified HPLC method. Clin Chim Acta *252*, 83-93.
Easton, R. M., Deckwerth, T. L., Parsadanian, A. S., and Johnson, E. M., Jr. (1997). Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J Neurosci *17*, 9656-9666.
Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A. W. (1998). DNA methylation models histone acetylation. Nature *394*, 842.
Elliott, E. M., and Sapolsky, R. M. (1992). Corticosterone enhances kainic acid-induced calcium elevation in cultured hippocampal neurons. J Neurochem *59*, 1033-1040.
Elliott, E. M., and Sapolsky, R. M. (1993). Corticosterone impairs hippocampal neuronal calcium regulation-- possible mediating mechanisms. Brain Res *602*, 84-90.
Ellis, R. E., Yuan, J. Y., and Horvitz, H. R. (1991). Mechanisms and functions of cell death. Annu Rev Cell Biol *7*, 663-698.
Elwood, R. W. (1983). Parental Behaviour of Rodents (Norwich, United Kingdom: John Wiley & Sons Ltd., Printed by Page Bros. Ltd.).
Encio, I. J., and Detera-Wadleigh, S. D. (1991). The genomic structure of the human glucocorticoid receptor. J Biol Chem *266*, 7182-7188.
Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994). Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J Biol Chem *269*, 15520-15527.
Evan, G., and Littlewood, T. (1998). A matter of life and cell death. Science *281*, 1317-1322.
Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Evans, R. M., Weinberger, C., Hollenberg, S., Swanson, L., Nelson, C., and Rosenfeld, M. G. (1985). Inducible and developmental control of neuroendocrine genes. Cold Spring Harb Symp Quant Biol *50*, 389-397.
Evoniuk, G. E., Kuhn, C. M., and Schanberg, S. M. (1979). The effect of tactile stimulation on serum growth hormone and tissue ornithine decarboxylase activity during maternal deprivation in rat pups. Commun Psychopharmacol *3*, 363-370.
Fahrbach, S. E., Morrell, J. I., and Pfaff, D. W. (1984). Oxytocin induction of short-latency maternal behavior in nulliparous, estrogen-primed female rats. Horm Behav *18*, 267-286.
Fahrbach, S. E., Morrell, J. I., and Pfaff, D. W. (1985). Possible role for endogenous oxytocin in estrogen-facilitated maternal behavior in rats. Neuroendocrinology *40*, 526-532.
Farlie, P. G., Dringen, R., Rees, S. M., Kannourakis, G., and Bernard, O. (1995). bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc Natl Acad Sci U S A *92*, 4397-4401.
Fazio, C., Andreoli, V., Agnoli, A., Casacchia, M., and Cerbo, R. (1973). [Therapeutic effects and mechanism of action of S-adenosyl-L-methionine (SAM) in depressive syndromes]. Minerva Med *64*, 1515-1529.
Feldman, S., and Conforti, N. (1977). Adrenocortical responses to olfactory stimulation in rats with partial hypothalamic deafferentations and lesions. Neuroendocrinology *24*, 162-168.
Felig, D. M., Brusilow, S. W., and Boyer, J. L. (1995). Hyperammonemic coma due to parenteral nutrition in a woman with heterozygous ornithine transcarbamylase deficiency. Gastroenterology *109*, 282-284.
Fleming, A. (1986). Psychobiology of rat maternal behavior: how and where hormones act to promote maternal behavior at parturition. Ann N Y Acad Sci *474*, 234-251.
Fleming, A. S., O'Day, D. H., and Kraemer, G. W. (1999). Neurobiology of mother-infant interactions: experience and central nervous system plasticity across development and generations. Neurosci Biobehav Rev *23*, 673-685.
Fleming, A. S., and Rosenblatt, J. S. (1974a). Maternal behavior in the virgin and lactating rat. J Comp Physiol Psychol *86*, 957-972.
Fleming, A. S., and Rosenblatt, J. S. (1974b). Olfactory regulation of maternal behavior in rats. I. Effects of olfactory bulb removal in experienced and inexperienced lactating and cycling females. J Comp Physiol Psychol *86*, 221-232.
Fleming, A. S., and Rosenblatt, J. S. (1974c). Olfactory regulation of maternal behavior in rats. II. Effects of peripherally induced anosmia and lesions of the lateral olfactory tract in pup-induced virgins. J Comp Physiol Psychol *86*, 233-246.
Foy, M. R., Stanton, M. E., Levine, S., and Thompson, R. F. (1987). Behavioral stress impairs long-term potentiation in rodent hippocampus. Behav Neural Biol *48*, 138-149.
Francis, D., Diorio, J., Liu, D., and Meaney, M. J. (1999). Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science *286*, 1155-1158.
Francis, D. D., Szegda, K., Campbell, G., Martin, W. D., and Insel, T. R. (2003). Epigenetic sources of behavioral differences in mice. Nat Neurosci *6*, 445-446.
Frank, E., and Thase, M. E. (1999). Natural history and preventative treatment of recurrent mood disorders. Annu Rev Med *50*, 453-468.
Frankova, S. (1985). Maternal behavior in females of the laboratory rat selected for high and low activity and defecation rates. Act Nerv Super (Praha) *27*, 186-198.
Friso, S., Choi, S. W., Girelli, D., Mason, J. B., Dolnikowski, G. G., Bagley, P. J., Olivieri, O., Jacques, P. F., Rosenberg, I. H., Corrocher, R., and Selhub, J. (2002). A common muta-

tion in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A *99*, 5606-5611.
Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., and Paul, C. L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A *89*, 1827-1831.
Funder, J. W. (1989). Hormonal regulation of gene expression. Biochem Soc Symp *55*, 105-114.
Fuster, J. M. (1995). Memory in the Cerebral Cortex: An Empirical Approach to Neural Networks in the Human and Nonhuman Primate. (Massachusetts: The MIT Press).
Gale, C. R., and Martyn, C. N. (1996). Breastfeeding, dummy use, and adult intelligence. Lancet *347*, 1072-1075.
Gall, C., and Lynch, G. (1980). The regulation of fiber growth and synaptogenesis in the developing hippocampus. Curr Top Dev Biol *15*, 159-180.
Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. J Mol Biol *196*, 261-282.
Gartler, S. M., and Riggs, A. D. (1983). Mammalian X-chromosome inactivation. Annu Rev Genet *17*, 155-190.
Gauguier, D., Froguel, P., Parent, V., Bernard, C., Bihoreau, M. T., Portha, B., James, M. R., Penicaud, L., Lathrop, M., and Ktorza, A. (1996). Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. Nat Genet *12*, 38-43.
Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., *et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol *5*, R80.
Ghorbel, M. T., Seugnet, I., Hadj-Sahraoui, N., Topilko, P., Levi, G., and Demeneix, B. (1999). Thyroid hormone effects on Krox-24 transcription in the post-natal mouse brain are developmentally regulated but are not correlated with mitosis. Oncogene *18*, 917-924.
Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645-652.
Glass, C. K. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev *15*, 391-407.
Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997). Nuclear receptor coactivators. Curr Opin Cell Biol *9*, 222-232.
Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature *325*, 365-368.
Goldner-Sauve, A., Szpirer, C., Szpirer, J., Levan, G., and Gasser, D. L. (1991). Chromosome assignments of the genes for glucocorticoid receptor, myelin basic protein, leukocyte common antigen, and TRPM2 in the rat. Biochem Genet *29*, 275-286.
Goldstein, D. S., Dionne, R., Sweet, J., Gracely, R., Brewer, H. B., Jr., Gregg, R., and Keiser, H. R. (1982). Circulatory, plasma catecholamine, cortisol, lipid, and psychological responses to a real-life stress (third molar extractions): effects of diazepam sedation and of inclusion of epinephrine with the local anesthetic. Psychosom Med *44*, 259-272.
Gonzalez, A., Lovic, V., Ward, G. R., Wainwright, P. E., and Fleming, A. S. (2001). Intergenerational effects of complete maternal deprivation and replacement stimulation on maternal behavior and emotionality in female rats. Dev Psychobiol *38*, 11-32.
Gonzalez, D. E., and Deis, R. P. (1986). Maternal behavior in cyclic and androgenized fe-

male rats: role of ovarian hormones. Physiol Behav *38*, 789-793.
Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, W., 3rd, Vale, W. W., and Montminy, M. R. (1989). A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature *337*, 749-752.
Gottlicher, M., Heck, S., and Herrlich, P. (1998). Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med *76*, 480-489.
Gould, E., Beylin, A., Tanapat, P., Reeves, A., and Shors, T. J. (1999a). Learning enhances adult neurogenesis in the hippocampal formation [see comments]. Nat Neurosci *2*, 260-265.
Gould, E., Tanapat, P., Hastings, N. B., and Shors, T. J. (1999b). Neurogenesis in adulthood: a possible role in learning. Trends in Cognitive Sciences *3*, 186-192.
Govindan, M. V., Pothier, F., Leclerc, S., Palaniswami, R., and Xie, B. (1991). Human glucocorticoid receptor gene promotor-homologous down regulation. J Steroid Biochem Mol Biol *40*, 317-323.
Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., Guidotti, A., and Costa, E. (2005). Reelin promoter hypermethylation in schizophrenia. Proc Natl Acad Sci U S A *102*, 9341-9346.
Green, D. R., and Reed, J. C. (1998). Mitochondria and apoptosis. Science *281*, 1309-1312.
Greene, L. A., and Kaplan, D. R. (1995). Early events in neurotrophin signalling via Trk and p75 receptors. Curr Opin Neurobiol *5*, 579-587.
Grody, W. W., Schrader, W. T., and O'Malley, B. W. (1982). Activation, transformation, and subunit structure of steroid hormone receptors. Endocr Rev *3*, 141-163.
Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*, 1899-1911.
Grota, L. J., Ader, R. (1969). Continuous recording of maternal behaviour in Rattus Norvegicus. Animal Behaviour, 722-729.
Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature *389*, 349-352.
Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., Thanos, D., and Kandel, E. R. (2002). Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. Cell *111*, 483-493.
Gubernick, D. J., and Alberts, J. R. (1983). Maternal licking of young: resource exchange and proximate controls. Physiol Behav *31*, 593-601.
Habener, J. F., Meyer, T. E., Yun, Y., Waeber, G., and Hoeffler, J. P. (1990). Characterization of a cAMP-regulated enhancer-binding protein. Metabolism *39*, 13-16.
Hammond, G. L., Smith, C. L., Paterson, N. A., and Sibbald, W. J. (1990). A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. J Clin Endocrinol Metab *71*, 34-39.
Hammond, G. L., Smith, C. L., and Underhill, D. A. (1991). Molecular studies of corticosteroid binding globulin structure, biosynthesis and function. J Steroid Biochem Mol Biol *40*, 755-762.
Hansen, S., Bergvall, A. H., and Nyiredi, S. (1993). Interaction with pups enhances dopamine release in the ventral striatum of maternal rats: a microdialysis study. Pharmacol Biochem Behav *45*, 673-676.
Harikrishnan, K. N., Chow, M. Z., Baker, E. K., Pal, S., Bassal, S., Brasacchio, D., Wang, L., Craig, J. M., Jones, P. L., Sif, S., and El-Osta, A. (2005). Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. Nat

Genet *37*, 254-264.
Hashimshony, T., Zhang, J., Keshet, I., Bustin, M., and Cedar, H. (2003). The role of DNA methylation in setting up chromatin structure during development. Nat Genet *34*, 187-192.
Heby, O., and Emanuelsson, H. (1981). Role of the polyamines in germ cell differentiation and in early embryonic development. Med Biol *59*, 417-422.
Hendry, I. A., Stockel, K., Thoenen, H., and Iversen, L. L. (1974). The retrograde axonal transport of nerve growth factor. Brain Res *68*, 103-121.
Herman, J. P. (1993). Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. Cell Mol Neurobiol *13*, 349-372.
Herman, J. P., Patel, P. D., Akil, H., and Watson, S. J. (1989). Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. Mol Endocrinol *3*, 1886-1894.
Hershko, A. Y., Kafri, T., Fainsod, A., and Razin, A. (2003). Methylation of HoxA5 and HoxB5 and its relevance to expression during mouse development. Gene *302*, 65-72.
Hilbert, P., Lindpaintner, K., Beckmann, J. S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., De Gouyon, B., Julier, C., Takahasi, S., and et al. (1991). Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. Nature *353*, 521-529.
Hoess, R. H., and Abremski, K. (1985). Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. J Mol Biol *181*, 351-362.
Hofer, M. A. (1994). Early relationships as regulators of infant physiology and behavior. Acta Paediatr Suppl *397*, 9-18.
Hofer, M. A. (1996). Multiple regulators of ultrasonic vocalization in the infant rat. Psychoneuroendocrinology *21*, 203-217.
Hollenberg, S. M., Giguere, V., Segui, P., and Evans, R. M. (1987). Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell *49*, 39-46.
Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature *318*, 635-641.
Hong, L., Schroth, G. P., Matthews, H. R., Yau, P., and Bradbury, E. M. (1993). Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 "tail" to DNA. J Biol Chem *268*, 305-314.
Horike, S., Cai, S., Miyano, M., Cheng, J. F., and Kohwi-Shigematsu, T. (2005). Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. Nat Genet *37*, 31-40.
Housley, P. R., Sanchez, E. R., Danielsen, M., Ringold, G. M., and Pratt, W. B. (1990). Evidence that the conserved region in the steroid binding domain of the glucocorticoid receptor is required for both optimal binding of hsp90 and protection from proteolytic cleavage. A two-site model for hsp90 binding to the steroid binding domain. J Biol Chem *265*, 12778-12781.
Hout, R. L., Ladd, C. O., and Plotsky, P. M. (2000). Maternal deprivation, In Encyclopedia of Stress, G. Fink, ed. (San Diego, CA, USA: Academic Press), pp. 699-707.
Huang, Y., Doherty, J. J., and Dingledine, R. (2002). Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. J Neurosci *22*, 8422-8428.

Humblot, N., Esteve, L., Burgun, C., Aunis, D., and Zwiller, J. (1997). 5-Hydroxytryptamine induces TIS8/egr-1 and c-fos expression in PC12 cells. Involvement of tyrosine protein phosphorylation. Eur J Neurosci *9*, 84-92.
Hutchison, K. A., Scherrer, L. C., Czar, M. J., Stancato, L. F., Chow, Y. H., Jove, R., and Pratt, W. B. (1993). Regulation of glucocorticoid receptor function through assembly of a receptor-heat shock protein complex. Ann N Y Acad Sci *684*, 35-48.
Hyman, S. E., Comb, M., Lin, Y. S., Pearlberg, J., Green, M. R., and Goodman, H. M. (1988). A common trans-acting factor is involved in transcriptional regulation of neurotransmitter genes by cyclic AMP. Mol Cell Biol *8*, 4225-4233.
Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics *4*, 249-264.
Iwasaki, K., Chung, E. H., and Fujiwara, M. (1998). [Apoptosis in the repeated cerebral ischemia--behavioral & histochemical study]. Nippon Yakurigaku Zasshi *112 Suppl 1*, 88P-92P.
Jacob, H. J., Brown, D. M., Bunker, R. K., Daly, M. J., Dzau, V. J., Goodman, A., Koike, G., Kren, V., Kurtz, T., Lernmark, A., and et al. (1995). A genetic linkage map of the laboratory rat, Rattus norvegicus. Nat Genet *9*, 63-69.
Jacobson, L., and Sapolsky, R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic- pituitary-adrenocortical axis. Endocr Rev *12*, 118-134.
Jefcoate, C. R., McNamara, B. C., and DiBartolomeis, M. J. (1986). Control of steroid synthesis in adrenal fasciculata cells. Endocr Res *12*, 315-350.
Joels, M., and de Kloet, E. R. (1989). Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. Science *245*, 1502-1505.
Johansson-Wallsten, C. E., Berg, M., and Meyerson, B. J. (1993). The effects of long-term treatment with the 5-HT1A receptor agonist 8-OH-DPAT and the 5-HT2/1C receptor agonist DOI in the neonatal rat. Eur J Pharmacol *243*, 149-154.
Jones, P. A., and Laird, P. W. (1999). Cancer epigenetics comes of age. Nat Genet *21*, 163-167.
Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet *19*, 187-191.
Juliano, R. L., Dixit, V. R., Kang, H., Kim, T. Y., Miyamoto, Y., and Xu, D. (2005). Epigenetic manipulation of gene expression: a toolkit for cell biologists. J Cell Biol *169*, 847-857.
Jutapakdeegul, N., Casalotti, S. O., Govitrapong, P., and Kotchabhakdi, N. (2003). Postnatal touch stimulation acutely alters corticosterone levels and glucocorticoid receptor gene expression in the neonatal rat. Dev Neurosci *25*, 26-33.
Kadar, T., Silbermann, M., Brandeis, R., and Levy, A. (1990). Age-related structural changes in the rat hippocampus: correlation with working memory deficiency. Brain Res *512*, 113-120.
Kadonaga, J. T. (1998). Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell *92*, 307-313.
Kadonaga, J. T., and Tjian, R. (1986). Affinity purification of sequence-specific DNA binding proteins. Proc Natl Acad Sci U S A *83*, 5889-5893.
Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., and Razin, A. (1992). Developmental pattern of gene-specific DNA methylation in the mouse

embryo and germ line. Genes Dev *6*, 705-714.
Kafri, T., Gao, X., and Razin, A. (1993). Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo. Proc Natl Acad Sci U S A *90*, 10558-10562.
Kajiwara, R., Sand, O., Kidokoro, Y., Barish, M. E., and Iijima, T. (1997). Functional organization of chromaffin cells and cholinergic synaptic transmission in rat adrenal medulla. Jpn J Physiol *47*, 449-464.
Kalcheim, C., Carmeli, C., and Rosenthal, A. (1992). Neurotrophin 3 is a mitogen for cultured neural crest cells. Proc Natl Acad Sci U S A *89*, 1661-1665.
Kalynchuk, L. E., and Meaney, M. J. (2003). Amygdala kindling increases fear responses and decreases glucocorticoid receptor mRNA expression in hippocampal regions. Prog Neuropsychopharmacol Biol Psychiatry *27*, 1225-1234.
Karalis, K., Muglia, L. J., Bae, D., Hilderbrand, H., and Majzoub, J. A. (1997). CRH and the immune system. J Neuroimmunol *72*, 131-136.
Karanth, S., Linthorst, A. C., Stalla, G. K., Barden, N., Holsboer, F., and Reul, J. M. (1997). Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. Endocrinology *138*, 3476-3485.
Keller-Wood, M., Shinsako, J., and Dallman, M. F. (1984). Interaction between stimulus intensity and corticosteroid feedback in control of ACTH. Am J Physiol *247*, E489-494.
Kempermann, G., and Gage, F. H. (1999). Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal. Hippocampus *9*, 321-332.
Kerr, D. S., Campbell, L. W., Applegate, M. D., Brodish, A., and Landfield, P. W. (1991). Chronic stress-induced acceleration of electrophysiologic and morphometric biomarkers of hippocampal aging. J Neurosci *11*, 1316-1324.
Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.
Keshet, I., Yisraeli, J., and Cedar, H. (1985). Effect of regional DNA methylation on gene expression. Proc Natl Acad Sci U S A *82*, 2560-2564.
Kirillov, A., Kistler, B., Mostoslavsky, R., Cedar, H., Wirth, T., and Bergman, Y. (1996). A role for nuclear NF-kappaB in B-cell-specific demethylation of the Igkappa locus. Nat Genet *13*, 435-441.
Kirkwood, A., Dudek, S. M., Gold, J. T., Aizenman, C. D., and Bear, M. F. (1993). Common forms of synaptic plasticity in the hippocampus and neocortex in vitro. Science *260*, 1518-1521.
Klug, A. (1995). Gene regulatory proteins and their interaction with DNA. Ann N Y Acad Sci *758*, 143-160.
Koide, Y., Kimura, S., Inoue, S., Ikeda, M., Uchida, K., Ando, J., Shimizu, A., Oda, K., Itakura, M., Nabeshima, I., and et al. (1986). Responsiveness of hypophyseal-adrenocortical axis to repetitive administration of synthetic ovine corticotropin-releasing hormone in patients with isolated adrenocorticotropin deficiency. J Clin Endocrinol Metab *63*, 329-335.
Konradi, C., Cole, R. L., Heckers, S., and Hyman, S. E. (1994). Amphetamine regulates gene expression in rat striatum via transcription factor CREB. J Neurosci *14*, 5623-5634.
Kornberg, R. D. (1974). Chromatin structure: a repeating unit of histones and DNA. Science *184*, 868-871.
Korsmeyer, S. J., Shutter, J. R., Veis, D. J., Merry, D. E., and Oltvai, Z. N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol

4, 327-332.
Kovacs, P., Voigt, B., and Kloting, I. (1997). Novel quantitative trait loci for blood pressure and related traits on rat chromosomes 1, 10, and 18. Biochem Biophys Res Commun *235*, 343-348.
Kraemer, G. W. (1997). Psychobiology of early social attachment in rhesus monkeys. Clinical implications. Ann N Y Acad Sci *807*, 401-418.
Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res *53*, 4701-4714.
Krinke, G. J. (2000). The Laboratory Rat (San Diego, CA, USA: Academic Press).
Kroemer, G., Zamzami, N., and Susin, S. A. (1997). Mitochondrial control of apoptosis. Immunol Today *18*, 44-51.
Kuhn, C. M., Butler, S. R., and Schanberg, S. M. (1978). Selective depression of serum growth hormone during maternal deprivation in rat pups. Science *201*, 1034-1036.
Kuhn, C. M., Evoniuk, G., and Schanberg, S. M. (1979). Loss of tissue sensitivity to growth hormone during maternal deprivation in rats. Life Sci *25*, 2089-2097.
Kuhnert, B. R., Knapp, D. R., Kuhnert, P. M., and Prochaska, A. L. (1979). Maternal, fetal, and neonatal metabolism of lidocaine. Clin Pharmacol Ther *26*, 213-220.
Kukita, T., Kukita, A., Harada, H., and Iijima, T. (1997). Regulation of osteoclastogenesis by antisense oligodeoxynucleotides specific to zinc finger nuclear transcription factors Egr-1 and WT1 in rat bone marrow culture system. Endocrinology *138*, 4384-4389.
Kumar, A., Commane, M., Flickinger, T. W., Horvath, C. M., and Stark, G. R. (1997). Defective TNF-alpha-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science *278*, 1630-1632.
Kurreck, J. (2004). Antisense and RNA interference approaches to target validation in pain research. Curr Opin Drug Discov Devel *7*, 179-187.
Kvetnansky, R., Pacak, K., Fukuhara, K., Viskupic, E., Hiremagalur, B., Nankova, B., Goldstein, D. S., Sabban, E. L., and Kopin, I. J. (1995). Sympathoadrenal system in stress. Interaction with the hypothalamic- pituitary-adrenocortical system. Ann N Y Acad Sci *771*, 131-158.
Ladd, C. O., Huot, R. L., Thrivikraman, K. V., Nemeroff, C. B., Meaney, M. J., and Plotsky, P. M. (2000). Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog Brain Res *122*, 81-103.
Laird, P. W., and Jaenisch, R. (1996). The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet *30*, 441-464.
Landers, J. P., and Spelsberg, T. C. (1992). New concepts in steroid hormone action: transcription factors, proto- oncogenes, and the cascade model for steroid regulation of gene expression. Crit Rev Eukaryot Gene Expr *2*, 19-63.
Landfield, P. W., and Pitler, T. A. (1984). Prolonged Ca2+-dependent afterhyperpolarizations in hippocampal neurons of aged rats. Science *226*, 1089-1092.
Landfield, P. W., Rose, G., Sandles, L., Wohlstadter, T. C., and Lynch, G. (1977). Patterns of astroglial hypertrophy and neuronal degeneration in the hippocampus of ages, memory-deficient rats. J Gerontol *32*, 3-12.
Lane-Ladd, S. B., Pineda, J., Boundy, V. A., Pfeuffer, T., Krupinski, J., Aghajanian, G. K., and Nestler, E. J. (1997). CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate depend-

ence. J Neurosci *17*, 7890-7901.
Laplante, P., Diorio, J., and Meaney, M. J. (2002). Serotonin regulates hippocampal glucocorticoid receptor expression via a 5-HT7 receptor. Brain Res Dev Brain Res *139*, 199-203.
Laudet, V., Hanni, C., Coll, J., Catzeflis, F., and Stehelin, D. (1992). Evolution of the nuclear receptor gene superfamily. Embo J *11*, 1003-1013.
LeDoux, J. E., Cicchetti, P., Xagoraris, A., and Romanski, L. M. (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J Neurosci *10*, 1062-1069.
Lehmann, J., and Feldon, J. (2000). Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? Rev Neurosci *11*, 383-408.
Leon, M., Croskerry, P. G., and Smith, G. K. (1978). Thermal control of mother-young contact in rats. Physiol Behav *21*, 79O-811.
Leonhardt, H., and Bestor, T. H. (1993). Structure, function and regulation of mammalian DNA methyltransferase. Exs *64*, 109-119.
Levine, E. S., Crozier, R. A., Black, I. B., and Plummer, M. R. (1998). Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci U S A *95*, 10235-10239.
Levine, S. (1967). Maternal and environmental influences on the adrenocortical response to stress in weanling rats. Science *156*, 258-260.
Levine, S. (1994). The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. Ann N Y Acad Sci *746*, 275-288; discussion 289-293.
Levine, S. (2002). Enduring effects of early experience on adult behavior, In Hormones, brain and behavior, D. W. Pfaff, A. P. Arnold, S. E. Fahrbach, A. M. Etgen, and R. T. Rubin, eds. (San Diego, CA, USA: Academic Press), pp. 535-542.
Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet *3*, 662-673.
Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-926.
Li, S., Givalois, L., and Pelletier, G. (1997). Involvement of neurosteroids in the effect of the endogenous benzodiazepine receptor ligand octadecaneuropeptide (ODN) on gonadotropin-releasing hormone gene expression in rat brain. J Neuroendocrinol *9*, 229-233.
Liu, D., Diorio, J., Day, J. C., Francis, D. D., and Meaney, M. J. (2000). Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosci *3*, 799-806.
Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P. M., and Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic- pituitary-adrenal responses to stress [see comments]. Science *277*, 1659-1662.
Lopez, E., Pozas, E., Rivera, R., and Ferrer, I. (1999a). Bcl-2 and Bax expression following methylazoxymethanol acetate-induced apoptosis in the external granule cell layer of the developing rat cerebellum. Brain Res Dev Brain Res *112*, 149-153.
Lopez, E., Pozas, E., Rivera, R., and Ferrer, I. (1999b). Bcl-2, Bax and Bcl-x expression following kainic acid administration at convulsant doses in the rat. Neuroscience *91*, 1461-1470.
Loven, M. A., Wood, J. R., and Nardulli, A. M. (2001). Interaction of estrogen receptors alpha and beta with estrogen response elements. Mol Cell Endocrinol *181*, 151-163.
Lovenberg, T. W., Baron, B. M., de Lecea, L., Miller, J. D., Prosser, R. A., Rea, M. A., Foye, P. E., Racke, M., Slone, A. L., Siegel, B. W., and et al. (1993a). A novel adenylyl

cyclase-activating serotonin receptor (5-HT7) implicated in the regulation of mammalian circadian rhythms. Neuron *11*, 449-458.
Lovenberg, T. W., Erlander, M. G., Baron, B. M., and Sutcliffe, J. G. (1993b). Cloning of new 5-HT receptors. Int Clin Psychopharmacol *8 Suppl 2*, 19-23.
Lucas, A. (1998). Programming by early nutrition: an experimental approach. J Nutr *128*, 401S-406S.
Luckman, S. M., and Cox, H. J. (1995). Expression of inducible cAMP early repressor (ICER) in hypothalamic magnocellular neurons. Brain Res Mol Brain Res *34*, 231-238.
Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature *352*, 497-505.
Maccari, S., Piazza, P. V., Kabbaj, M., Barbazanges, A., Simon, H., and Le Moal, M. (1995). Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J Neurosci *15*, 110-116.
Macleod, D., Charlton, J., Mullins, J., and Bird, A. P. (1994). Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev *8*, 2282-2292.
Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and et al. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.
Marin, M. C., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J., and McDonnell, T. J. (1996). Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+. Oncogene *12*, 2259-2266.
Martinou, J. C., Dubois-Dauphin, M., Staple, J. K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C., and et al. (1994). Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron *13*, 1017-1030.
Martins, V. R., Pratt, W. B., Terracio, L., Hirst, M. A., Ringold, G. M., and Housley, P. R. (1991). Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed in a nonrandom manner throughout all planes of the nucleus. Mol Endocrinol *5*, 217-225.
Marton, L. J. (1987). Effects of treatment with DNA-directed cancer chemotherapeutic agents after polyamine depletion. Pharmacol Ther *32*, 183-190.
Matsuoka, I. (1994). [Receptors and signal transduction mechanisms in olfaction and taste]. Seikagaku *66*, 150-154.
Mayer, A. D., and Rosenblatt, J. S. (1975). Olfactory basis for the delayed onset of maternal behavior in virgin female rats: experimential effects. J Comp Physiol Psychol *89*, 701-710.
McCarty, R., and Lee, J. H. (1996). Maternal influences on adult blood pressure of SHRs: a single pup cross-fostering study. Physiol Behav *59*, 71-75.
McCormick, J. A., Lyons, V., Jacobson, M. D., Noble, J., Diorio, J., Nyirenda, M., Weaver, S., Ester, W., Yau, J. L., Meaney, M. J., *et al.* (2000). 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. Mol Endocrinol *14*, 506-517.
McEwen, B. S. (1996). Gonadal and adrenal steroids regulate neurochemical and structural plasticity of the hippocampus via cellular mechanisms involving NMDA receptors. Cell

Mol Neurobiol *16*, 103-116.
McEwen, B. S. (1997). Hormones as regulators of brain development: life-long effects related to health and disease. Acta Paediatr Suppl *422*, 41-44.
Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci *24*, 1161-1192.
Meaney, M. J., and Aitken, D. H. (1985). The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations: temporal parameters. Brain Res *354*, 301-304.
Meaney, M. J., Aitken, D. H., and Sapolsky, R. M. (1987). Thyroid hormones influence the development of hippocampal glucocorticoid receptors in the rat: a mechanism for the effects of postnatal handling on the development of the adrenocortical stress response. Neuroendocrinology *45*, 278-283.
Meaney, M. J., Aitken, D. H., van Berkel, C., Bhatnagar, S., and Sapolsky, R. M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. Science *239*, 766-768.
Meaney, M. J., Aitken, D. H., Viau, V., Sharma, S., and Sarrieau, A. (1989). Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology *50*, 597-604.
Meaney, M. J., Diorio, J., Francis, D., Weaver, S., Yau, J., Chapman, K., and Seckl, J. R. (2000). Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. J Neurosci *20*, 3926-3935.
Meaney, M. J., Diorio, J., Francis, D., Widdowson, J., LaPlante, P., Caldji, C., Sharma, S., Seckl, J. R., and Plotsky, P. M. (1996). Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. Dev Neurosci *18*, 49-72.
Mei, S., Ho, A. D., and Mahlknecht, U. (2004). Role of histone deacetylase inhibitors in the treatment of cancer (Review). Int J Oncol *25*, 1509-1519.
Mello, C. C., and Conte, D., Jr. (2004). Revealing the world of RNA interference. Nature *431*, 338-342.
Mellstrom, B., Naranjo, J. R., Foulkes, N. S., Lafarga, M., and Sassone-Corsi, P. (1993). Transcriptional response to cAMP in brain: specific distribution and induction of CREM antagonists. Neuron *10*, 655-665.
Mellstrom, B., Pipaon, C., Naranjo, J. R., Perez-Castillo, A., and Santos, A. (1994). Differential effect of thyroid hormone on NGFI-A gene expression in developing rat brain. Endocrinology *135*, 583-588.
Merry, D. E., Veis, D. J., Hickey, W. F., and Korsmeyer, S. J. (1994). bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development *120*, 301-311.
Michael, G. J., Kaya, E., Averill, S., Rattray, M., Clary, D. O., and Priestley, J. V. (1997). TrkA immunoreactive neurones in the rat spinal cord. J Comp Neurol *385*, 441-455.
Michaelidis, T. M., Sendtner, M., Cooper, J. D., Airaksinen, M. S., Holtmann, B., Meyer, M., and Thoenen, H. (1996). Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development. Neuron *17*, 75-89.
Miesfeld, R., Godowski, P. J., Maler, B. A., and Yamamoto, K. R. (1987). Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. Science *236*,

423-427.
Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikstrom, A. C., Gustafsson, J. A., and Yamamoto, K. R. (1986). Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell *46*, 389-399.
Milbrandt, J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science *238*, 797-799.
Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron *1*, 183-188.
Miller, J. W., and Crapo, L. (1993). The medical treatment of Cushing's syndrome. Endocr Rev *14*, 443-458.
Miller, J. W., Nadeau, M. R., Smith, J., Smith, D., and Selhub, J. (1994). Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. Biochem J *298 (Pt 2)*, 415-419.
Milner, T. A., Lee, A., Aicher, S. A., and Rosin, D. L. (1998). Hippocampal alpha2a-adrenergic receptors are located predominantly presynaptically but are also found post-synaptically and in selective astrocytes. J Comp Neurol *395*, 310-327.
Mitchell, J. B., Betito, K., Rowe, W., Boksa, P., and Meaney, M. J. (1992). Serotonergic regulation of type II corticosteroid receptor binding in hippocampal cell cultures: evidence for the importance of serotonin- induced changes in cAMP levels. Neuroscience *48*, 631-639.
Mitchell, J. B., Rowe, W., Boksa, P., and Meaney, M. J. (1990). Serotonin regulates type II corticosteroid receptor binding in hippocampal cell cultures. J Neurosci *10*, 1745-1752.
Mohammed, A. H., Henriksson, B. G., Soderstrom, S., Ebendal, T., Olsson, T., and Seckl, J. R. (1993). Environmental influences on the central nervous system and their implications for the aging rat. Behav Brain Res *57*, 183-191.
Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci U S A *83*, 6682-6686.
Moore, C. L. (1984). Maternal contributions to the development of masculine sexual behavior in laboratory rats. Dev Psychobiol *17*, 347-356.
Moore, C. L., and Morelli, G. A. (1979). Mother rats interact differently with male and female offspring. J Comp Physiol Psychol *93*, 677-684.
Moore, C. L., and Power, K. L. (1986). Prenatal stress affects mother-infant interaction in Norway rats. Dev Psychobiol *19*, 235-245.
Moore, C. L., and Power, K. L. (1992). Variation in maternal care and individual differences in play, exploration, and grooming of juvenile Norway rat offspring. Dev Psychobiol *25*, 165-182.
Moore, C. L., Wong, L., Daum, M. C., and Leclair, O. U. (1997). Mother-infant interactions in two strains of rats: implications for dissociating mechanism and function of a maternal pattern. Dev Psychobiol *30*, 301-312.
Moret, C. (2004). Impact of gene polymorphisms on depression and its treatment. IDrugs *7*, 558-562.
Morris, R. G., Garrud, P., Rawlins, J. N., and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. Nature *297*, 681-683.
Morse, J. K., and Davis, J. N. (1990). Regulation of ischemic hippocampal damage in the gerbil: adrenalectomy alters the rate of CA1 cell disappearance. Exp Neurol *110*, 86-92.
Mousseau, T. A., and Fox, C. W. (1998). The adaptive significance of maternal effects.

Trends Ecol Evol *13*, 403-407.
Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., *et al.* (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature *381*, 335-341.
Mudd, S. H., and Cantoni, G. L. (1958). Activation of methionine for transmethylation. III. The methionine-activating enzyme of Bakers' yeast. J Biol Chem *231*, 481-492.
Mummaneni, P., Bishop, P. L., and Turker, M. S. (1993). A cis-acting element accounts for a conserved methylation pattern upstream of the mouse adenine phosphoribosyltransferase gene. J Biol Chem *268*, 552-558.
Munck, A., and Foley, R. (1976). Kinetics of glucocorticoid-receptor complexes in rat thymus cells. J Steroid Biochem *7*, 1117-1122.
Munck, A., and Holbrook, N. J. (1984). Glucocorticoid-receptor complexes in rat thymus cells. Rapid kinetic behavior and a cyclic model. J Biol Chem *259*, 820-831.
Munck, A., and Holbrook, N. J. (1988). Cyclic, nonequilibrium models of glucocorticoid antagonism: role of activation, nuclear binding and receptor recycling. J Steroid Biochem *31*, 599-606.
Myers, M. M., Brunelli, S. A., Shair, H. N., Squire, J. M., and Hofer, M. A. (1989a). Relationships between maternal behavior of SHR and WKY dams and adult blood pressures of cross-fostered F1 pups. Dev Psychobiol *22*, 55-67.
Myers, M. M., Brunelli, S. A., Squire, J. M., Shindeldecker, R. D., and Hofer, M. A. (1989b). Maternal behavior of SHR rats and its relationship to offspring blood pressures. Dev Psychobiol *22*, 29-53.
Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-389.
Neame, S. J., Rubin, L. L., and Philpott, K. L. (1998). Blocking cytochrome c activity within intact neurons inhibits apoptosis. J Cell Biol *142*, 1583-1593.
Newmeyer, D. D., and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. Cell *52*, 641-653.
Newport, D. J., Stowe, Z. N., and Nemeroff, C. B. (2002). Parental depression: animal models of an adverse life event. Am J Psychiatry *159*, 1265-1283.
Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet *23*, 58-61.
Nicholls, D., and Attwell, D. (1990). The release and uptake of excitatory amino acids. Trends Pharmacol Sci *11*, 462-468.
Nicol, L. (1974). L'épopée Pastorienne et la Médicine Vétérinaire (Garches, France).
Nijhawan, D., Honarpour, N., and Wang, X. (2000). Apoptosis in neural development and disease. Annu Rev Neurosci *23*, 73-87.
Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. (2001). Mechanisms of estrogen action. Physiol Rev *81*, 1535-1565.
Noback, C. R., and Eisenman, L. M. (1981). Some effects of protein-calorie undernutrition on the developing central nervous system of the rat. Anat Rec *201*, 67-73.
Nobukuni, Y., Smith, C. L., Hager, G. L., and Detera-Wadleigh, S. D. (1995). Characterization of the human glucocorticoid receptor promoter. Biochemistry *34*, 8207-8214.
Numan, M. (1988). Neural basis of maternal behavior in the rat. Psychoneuroendocrinology

13, 47-62.
Numan, M., and Callahan, E. C. (1980). The connections of the medial preoptic region and maternal behavior in the rat. Physiol Behav *25*, 653-665.
Numan, M., and Numan, M. J. (1994). Expression of Fos-like immunoreactivity in the preoptic area of maternally behaving virgin and postpartum rats. Behav Neurosci *108*, 379-394.
Numan, M., and Smith, H. G. (1984). Maternal behavior in rats: evidence for the involvement of preoptic projections to the ventral tegmental area. Behav Neurosci *98*, 712-727.
Nunez, G., Benedict, M. A., Hu, Y., and Inohara, N. (1998). Caspases: the proteases of the apoptotic pathway. Oncogene *17*, 3237-3245.
Oakley, R. H., and Cidlowski, J. A. (1993). Homologous down regulation of the glucocorticoid receptor: the molecular machinery. Crit Rev Eukaryot Gene Expr *3*, 63-88.
Oltvai, Z. N., and Korsmeyer, S. J. (1994). Checkpoints of dueling dimers foil death wishes [comment]. Cell *79*, 189-192.
Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.
Ono, T., Luiten, P. G., Nishijo, H., Fukuda, M., and Nishino, H. (1985). Topographic organization of projections from the amygdala to the hypothalamus of the rat. Neurosci Res *2*, 221-238.
Oppenheim, R. W. (1991). Cell death during development of the nervous system. Annu Rev Neurosci *14*, 453-501.
Ormerod, B. K., and Galea, L. A. (2001). Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience *102*, 369-379.
Orti, E., Bodwell, J. E., and Munck, A. (1992). Phosphorylation of steroid hormone receptors. Endocr Rev *13*, 105-128.
Orti, E., Mendel, D. B., Smith, L. I., Bodwell, J. E., and Munck, A. (1989). A dynamic model of glucocorticoid receptor phosphorylation and cycling in intact cells. J Steroid Biochem *34*, 85-96.
Ottinger, D. R., and Tanabe, G. (1969). Maternal food restriction: effects on offspring behavior and development. Dev Psychobiol *2*, 7-9.
Pabo, C. O., Peisach, E., and Grant, R. A. (2001). Design and selection of novel Cys2His2 zinc finger proteins. Annu Rev Biochem *70*, 313-340.
Pascale, R. M., Simile, M. M., Satta, G., Seddaiu, M. A., Daino, L., Pinna, G., Vinci, M. A., Gaspa, L., and Feo, F. (1991). Comparative effects of L-methionine, S-adenosyl-L-methionine and 5'-methylthioadenosine on the growth of preneoplastic lesions and DNA methylation in rat liver during the early stages of hepatocarcinogenesis. Anticancer Res *11*, 1617-1624.
Pauk, J., Kuhn, C. M., Field, T. M., and Schanberg, S. M. (1986). Positive effects of tactile versus kinesthetic or vestibular stimulation on neuroendocrine and ODC activity in maternally-deprived rat pups. Life Sci *39*, 2081-2087.
Pavletich, N. P., and Pabo, C. O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science *252*, 809-817.
Paxinos, G., and Watson, C. (1996). The Rat Brain in Stereotaxic Coordinates (San Diego: Academic Press).
Pedersen, C. A., Caldwell, J. D., Johnson, M. F., Fort, S. A., and Prange, A. J., Jr. (1985). Oxytocin antiserum delays onset of ovarian steroid-induced maternal behavior. Neuropep-

tides *6*, 175-182.
Pedersen, C. A., and Prange, A. J., Jr. (1979). Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. Proc Natl Acad Sci U S A *76*, 6661-6665.
Pesold, C., Impagnatiello, F., Pisu, M. G., Uzunov, D. P., Costa, E., Guidotti, A., and Caruncho, H. J. (1998). Reelin is preferentially expressed in neurons synthesizing gamma-aminobutyric acid in cortex and hippocampus of adult rats. Proc Natl Acad Sci U S A *95*, 3221-3226.
Peters, D. A. (1982). Prenatal stress: effects on brain biogenic amine and plasma corticosterone levels. Pharmacol Biochem Behav *17*, 721-725.
Pfaff, D. W., Silva, M. T., and Weiss, J. M. (1971). Telemetered recording of hormone effects on hippocampal neurons. Science *172*, 394-395.
Pfeifer, G. P., Steigerwald, S. D., Hansen, R. S., Gartler, S. M., and Riggs, A. D. (1990). Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. Proc Natl Acad Sci U S A *87*, 8252-8256.
Picard, D., Salser, S. J., and Yamamoto, K. R. (1988). A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell *54*, 1073-1080.
Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J *6*, 3333-3340.
Pignatelli, D., Pinto, P., Magalhaes, M. M., and Magalhaes, M. C. (1998). The development of the adrenal gland zona glomerulosa in the rat. A morphological, immunohistochemical and biochemical study. Mol Cell Endocrinol *140*, 163-168.
Pinna, G., Agis-Balboa, R. C., Doueiri, M. S., Guidotti, A., and Costa, E. (2004). Brain neurosteroids in gender-related aggression induced by social isolation. Crit Rev Neurobiol *16*, 75-82.
Pipaon, C., Santos, A., and Perez-Castillo, A. (1992). Thyroid hormone up-regulates NGFI-A gene expression in rat brain during development. J Biol Chem *267*, 21-23.
Plagemann, P. G., and Erbe, J. (1976). Glucocorticoids--uptake by simple diffusion by cultured Reuber and Novikoff rat hepatoma cells. Biochem Pharmacol *25*, 1489-1494.
Plassat, J. L., Amlaiky, N., and Hen, R. (1993). Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Mol Pharmacol *44*, 229-236.
Plotsky, P. M. (1987). Regulation of hypophysiotropic factors mediating ACTH secretion. Ann N Y Acad Sci *512*, 205-217.
Plotsky, P. M., and Meaney, M. J. (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res *18*, 195-200.
Poirier, L. A., Wise, C. K., Delongchamp, R. R., and Sinha, R. (2001). Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer Epidemiol Biomarkers Prev *10*, 649-655.
Porsolt, R. D., Anton, G., Blavet, N., and Jalfre, M. (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol *47*, 379-391.
Porsolt, R. D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.
Power, K. L., and Moore, C. L. (1986). Prenatal stress eliminates differential maternal attention to male offspring in Norway rats. Physiol Behav *38*, 667-671.

Pratt, W. B. (1992). Control of steroid receptor function and cytoplasmic-nuclear transport by heat shock proteins. Bioessays *14*, 841-848.
Pratt, W. B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J Biol Chem *268*, 21455-21458.
Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M. G., Evans, R. M., and Baulieu, E. E. (1988). A region in the steroid binding domain determines formation of the non- DNA-binding, 9 S glucocorticoid receptor complex. J Biol Chem *263*, 267-273.
Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C., and Welsh, M. J. (1989). Interaction of the glucocorticoid receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation. Cancer Res *49*, 2222s-2229s.
Pratt, W. B., and Welsh, M. J. (1994). Chaperone functions of the heat shock proteins associated with steroid receptors. Semin Cell Biol *5*, 83-93.
Pravenec, M., Gauguier, D., Schott, J. J., Buard, J., Kren, V., Bila, V., Szpirer, C., Szpirer, J., Wang, J. M., Huang, H., *et al.* (1996). A genetic linkage map of the rat derived from recombinant inbred strains. Mamm Genome *7*, 117-127.
Pryce, C. R., Bettschen, D., and Feldon, J. (2001). Comparison of the effects of early handling and early deprivation on maternal care in the rat. Dev Psychobiol *38*, 239-251.
Pryce, C. R., and Feldon, J. (2003). Long-term neurobehavioural impact of the postnatal environment in rats: manipulations, effects and mediating mechanisms. Neurosci Biobehav Rev *27*, 57-71.
Qvarnstrom, A., and Price, T. D. (2001). Maternal effects, paternal effects and sexual selection. Trends Ecol Evol *16*, 95-100.
Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999). DNA methylation is a reversible biological signal. Proc Natl Acad Sci U S A *96*, 6107-6112.
Rastinejad, F., Perlmann, T., Evans, R. M., and Sigler, P. B. (1995). Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature *375*, 203-211.
Razin, A. (1998). CpG methylation, chromatin structure and gene silencing-a three-way connection. Embo J *17*, 4905-4908.
Razin, A., and Cedar, H. (1977). Distribution of 5-methylcytosine in chromatin. Proc Natl Acad Sci U S A *74*, 2725-2728.
Razin, A., and Cedar, H. (1993). DNA methylation and embryogenesis. Exs *64*, 343-357.
Razin, A., and Kafri, T. (1994). DNA methylation from embryo to adult. Prog Nucleic Acid Res Mol Biol *48*, 53-81.
Razin, A., and Szyf, M. (1984). DNA methylation patterns. Formation and function. Biochim Biophys Acta *782*, 331-342.
Reagan, L. P., and McEwen, B. S. (1997). Controversies surrounding glucocorticoid-mediated cell death in the hippocampus. J Chem Neuroanat *13*, 149-167.
Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-776.
Reed, J. C., Zha, H., Aime-Sempe, C., Takayama, S., and Wang, H. G. (1996). Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol *406*, 99-112.
Reichardt, H. M., and Schutz, G. (1998). Glucocorticoid signalling--multiple variations of a common theme. Mol Cell Endocrinol *146*, 1-6.
Reichardt, H. M., Tronche, F., Bauer, A., and Schutz, G. (2000). Molecular genetic analysis of glucocorticoid signaling using the Cre/loxP system. Biol Chem *381*, 961-964.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science *293*, 1089-1093.
Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the RAR-gamma ligand-binding domain bound to all- trans retinoic acid. Nature *378*, 681-689.
Ressler, R. H., and Anderson, L. T. (1973). Avoidance conditioning in mice as a function of their mothers' exposure to shock. Dev Psychobiol *6*, 105-111.
Reul, J. M., and de Kloet, E. R. (1985). Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology *117*, 2505-2511.
Rivier, C., Rivier, J., and Vale, W. (1986). Stress-induced inhibition of reproductive functions: role of endogenous corticotropin-releasing factor. Science *231*, 607-609.
Robbins, T. W., and Everitt, B. J. (1996). Neurobehavioural mechanisms of reward and motivation. Curr Opin Neurobiol *6*, 228-236.
Rosch, P. J. (1999). Reminiscences of Hans Selye, and the birth of "stress". Int J Emerg Ment Health *1*, 59-66.
Rosenblatt, J. S. (1967). Nonhormonal basis of maternal behavior in the rat. Science *156*, 1512-1514.
Rosenblatt, J. S. (1989). Ethology in the laboratory: reproductive behavior with special reference to maternal care among the mammals, In Ethoexperimental Approaches to the study of Behavior, R. J. Blanchard, P. F. Brain, D. C. Blanchard, and S. Parmigiani, eds. (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 288-300.
Rossiter, M. C. (1999). Maternal Effects as Adaptations (London: Oxford University Press).
Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone Acetyltransferases. Annu Rev Biochem *70*, 81-120.
Rouleau, J., Tanigawa, G., and Szyf, M. (1992). The mouse DNA methyltransferase 5'-region. A unique housekeeping gene promoter. J Biol Chem *267*, 7368-7377.
Rousseau, G. G. (1984). Control of gene expression by glucocorticoid hormones. Biochem J *224*, 1-12.
Rusconi, S., and Yamamoto, K. R. (1987). Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. Embo J *6*, 1309-1315.
Saito, S., Kobayashi, S., Ohashi, Y., Igarashi, M., Komiya, Y., and Ando, S. (1994). Decreased synaptic density in aged brains and its prevention by rearing under enriched environment as revealed by synaptophysin contents. J Neurosci Res *39*, 57-62.
Sapienza, C. (1990). Parental imprinting of genes. Sci Am *263*, 52-60.
Sapolsky, R. M. (1985). Glucocorticoid toxicity in the hippocampus: temporal aspects of neuronal vulnerability. Brain Res *359*, 300-305.
Sapolsky, R. M., Armanini, M. P., Packan, D. R., Sutton, S. W., and Plotsky, P. M. (1990). Glucocorticoid feedback inhibition of adrenocorticotropic hormone secretagogue release. Relationship to corticosteroid receptor occupancy in various limbic sites. Neuroendocrinology *51*, 328-336.
Sapolsky, R. M., Krey, L. C., and McEwen, B. S. (1985). Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. J Neurosci *5*, 1222-1227.
Sarrieau, A., Sharma, S., and Meaney, M. J. (1988). Postnatal development and environmental regulation of hippocampal glucocorticoid and mineralocorticoid receptors. Brain Res *471*, 158-162.
Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon,

H. S., Shuker, S. B., Chang, B. S., Minn, A. J., *et al.* (1997). Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science *275*, 983-986.
Schanberg, S. M., Evoniuk, G., and Kuhn, C. M. (1984). Tactile and nutritional aspects of maternal care: specific regulators of neuroendocrine function and cellular development. Proc Soc Exp Biol Med *175*, 135-146.
Schatzki, A., McMillian, M., and Miller, L. G. (1990). Glutamate, kainate and quisqualate enhance GABA-dependent chloride uptake in cortex. Brain Res Bull *25*, 239-243.
Scherrer, L. C., Picard, D., Massa, E., Harmon, J. M., Simons, S. S., Jr., Yamamoto, K. R., and Pratt, W. B. (1993). Evidence that the hormone binding domain of steroid receptors confers hormonal control on chimeric proteins by determining their hormone- regulated binding to heat-shock protein 90. Biochemistry *32*, 5381-5386.
Schibler, U., and Sierra, F. (1987). Alternative promoters in developmental gene expression. Annu Rev Genet *21*, 237-257.
Schmid, W., Cole, T. J., Blendy, J. A., and Schutz, G. (1995). Molecular genetic analysis of glucocorticoid signalling in development. J Steroid Biochem Mol Biol *53*, 33-35.
Schumacher, M., Guennoun, R., Robel, P., and Baulieu, E. E. (1997). Neurosteroids in the Hippocampus: Neuronal Plasticity and Memory. Stress *2*, 65-78.
Schwabe, J. W., Chapman, L., Finch, J. T., and Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell *75*, 567-578.
Sealy, L., and Chalkley, R. (1978). DNA associated with hyperacetylated histone is preferentially digested by DNase I. Nucleic Acids Res *5*, 1863-1876.
Seckl, J. R., French, K. L., O'Donnell, D., Meaney, M. J., Nair, N. P., Yates, C. M., and Fink, G. (1993). Glucocorticoid receptor gene expression is unaltered in hippocampal neurons in Alzheimer's disease. Brain Res Mol Brain Res *18*, 239-245.
Self, D. W., and Nestler, E. J. (1995). Molecular mechanisms of drug reinforcement and addiction. Annu Rev Neurosci *18*, 463-495.
Seress, L. (1998). Neuronal connections, cell formation and cell migration in the perinatal human hippocampal dentate gyrus. Cesk Fysiol *47*, 42-50.
Sharma, R. K., Wang, T. H., Wirch, E., and Wang, J. H. (1980). Purification and properties of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase. J Biol Chem *255*, 5916-5923.
Shen, Y., Monsma, F. J., Jr., Metcalf, M. A., Jose, P. A., Hamblin, M. W., and Sibley, D. R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. J Biol Chem *268*, 18200-18204.
Sheng, M., and Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron *4*, 477-485.
Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.
Shimizu, S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC [see comments]. Nature *399*, 483-487.
Silver, P. A. (1991). How proteins enter the nucleus. Cell *64*, 489-497.
Silverman, E. S., Du, J., Williams, A. J., Wadgaonkar, R., Drazen, J. M., and Collins, T. (1998). cAMP-response-element-binding-protein-binding protein (CBP) and p300 are transcriptional co-activators of early growth response factor-1 (Egr-1). Biochem J *336 (Pt 1)*,

183-189.
Slotkin, T. A., and Bartolome, J. (1986). Role of ornithine decarboxylase and the polyamines in nervous system development: a review. Brain Res Bull *17*, 307-320.
Slyshenkov, V. S., Shevalye, A. A., Liopo, A. V., and Wojtczak, L. (2002). Protective role of L-methionine against free radical damage of rat brain synaptosomes. Acta Biochim Pol *49*, 907-916.
Smith, C. L., and Hammond, G. L. (1989). Rat corticosteroid binding globulin: primary structure and messenger ribonucleic acid levels in the liver under different physiological conditions. Mol Endocrinol *3*, 420-426.
Smith, S. S. (1994). Biological implications of the mechanism of action of human DNA (cytosine-5)methyltransferase. Prog Nucleic Acid Res Mol Biol *49*, 65-111.
Smith, T. D., Adams, M. M., Gallagher, M., Morrison, J. H., and Rapp, P. R. (2000). Circuit-specific alterations in hippocampal synaptophysin immunoreactivity predict spatial learning impairment in aged rats. J Neurosci *20*, 6587-6593.
Smythe, J. W., Rowe, W. B., and Meaney, M. J. (1994). Neonatal handling alters serotonin (5-HT) turnover and 5-HT2 receptor binding in selected brain regions: relationship to the handling effect on glucocorticoid receptor expression. Brain Res Dev Brain Res *80*, 183-189.
Sousa, R. J., Tannery, N. H., and Lafer, E. M. (1989). In situ hybridization mapping of glucocorticoid receptor messenger ribonucleic acid in rat brain. Mol Endocrinol *3*, 481-494.
Squire, L. R. (1992). Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans [published erratum appears in Psychol Rev 1992 Jul;99(3):582]. Psychol Rev *99*, 195-231.
Stack, E. C., Balakrishnan, R., Numan, M. J., and Numan, M. (2002). A functional neuroanatomical investigation of the role of the medial preoptic area in neural circuits regulating maternal behavior. Behav Brain Res *131*, 17-36.
Stern, J. M. (1997). Offspring-induced nurturance: animal-human parallels. Dev Psychobiol *31*, 19-37.
Stern, J. M., and Johnson, S. K. (1990). Ventral somatosensory determinants of nursing behavior in Norway rats. I. Effects of variations in the quality and quantity of pup stimuli. Physiol Behav *47*, 993-1011.
Stern, J. M., and Kolunie, J. M. (1989). Perioral anesthesia disrupts maternal behavior during early lactation in Long-Evans rats. Behav Neural Biol *52*, 20-38.
Stohr, T., Schulte Wermeling, D., Szuran, T., Pliska, V., Domeney, A., Welzl, H., Weiner, I., and Feldon, J. (1998). Differential effects of prenatal stress in two inbred strains of rats. Pharmacol Biochem Behav *59*, 799-805.
Strahle, U., Schmidt, A., Kelsey, G., Stewart, A. F., Cole, T. J., Schmid, W., and Schutz, G. (1992). At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc Natl Acad Sci U S A *89*, 6731-6735.
Stratakis, C. A., and Chrousos, G. P. (1995). Neuroendocrinology and pathophysiology of the stress system. Ann N Y Acad Sci *771*, 1-18.
Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002). Genesis: cluster analysis of microarray data. Bioinformatics *18*, 207-208.
Suen, P. C., Wu, K., Levine, E. S., Mount, H. T., Xu, J. L., Lin, S. Y., and Black, I. B. (1997). Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic N-methyl-D-aspartate receptor subunit 1. Proc Natl Acad Sci U S A *94*, 8191-8195.
Sullivan, R. M., Shokrai, N., and Leon, M. (1988a). Physical stimulation reduces the body

temperature of infant rats. Dev Psychobiol *21*, 225-235.
Sullivan, R. M., Wilson, D. A., and Leon, M. (1988b). Physical stimulation reduces the brain temperature of infant rats. Dev Psychobiol *21*, 237-250.
Suomi, S. J. (1997). Early determinants of behaviour: evidence from primate studies. Br Med Bull *53*, 170-184.
Svaren, J., Ehrig, T., Abdulkadir, S. A., Ehrengruber, M. U., Watson, M. A., and Milbrandt, J. (2000). EGR1 target genes in prostate carcinoma cells identified by microarray analysis. J Biol Chem *275*, 38524-38531.
Szpirer, C., Szpirer, J., Van Vooren, P., Tissir, F., Simon, J. S., Koike, G., Jacob, H. J., Lander, E. S., Helou, K., Klinga-Levan, K., and Levan, G. (1998). Gene-based anchoring of the rat genetic linkage and cytogenetic maps: new regional localizations, orientation of the linkage groups, and insights into mammalian chromosome evolution. Mamm Genome *9*, 721-734.
Szyf, M. (2001). Towards a pharmacology of DNA methylation. Trends Pharmacol Sci *22*, 350-354.
Szyf, M., Pakneshan, P., and Rabbani, S. A. (2004). DNA methylation and breast cancer. Biochem Pharmacol *68*, 1187-1197.
Takahashi, L. K., Turner, J. G., and Kalin, N. H. (1992). Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats. Brain Res *574*, 131-137.
Takai, D., and Jones, P. A. (2003). The CpG island searcher: a new WWW resource. In Silico Biol *3*, 235-240.
Tao, X., Finkbeiner, S., Arnold, D. B., Shaywitz, A. J., and Greenberg, M. E. (1998). Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron *20*, 709-726.
Tate, P. H., and Bird, A. P. (1993). Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev *3*, 226-231.
Thiels, E., Alberts, J. R., and Cramer, C. P. (1990). Weaning in rats: II. Pup behavior patterns. Dev Psychobiol *23*, 495-510.
Thornberry, N. A. (1997). The caspase family of cysteine proteases. Br Med Bull *53*, 478-490.
Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-1316.
Tohda, M., and Watanabe, H. (2004). Molecular cloning and characterization of a novel sequence, vof-16, with enhanced expression in permanent ischemic rat brain. Biol Pharm Bull *27*, 1228-1235.
Toth, I. E., and Hinson, J. P. (1995). Neuropeptides in the adrenal gland: distribution, localization of receptors, and effects on steroid hormone synthesis. Endocr Res *21*, 39-51.
Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A *76*, 4350-4354.
Tremolizzo, L., Carboni, G., Ruzicka, W. B., Mitchell, C. P., Sugaya, I., Tueting, P., Sharma, R., Grayson, D. R., Costa, E., and Guidotti, A. (2002). An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability. Proc Natl Acad Sci U S A *99*, 17095-17100.
Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R., and Schutz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous

system results in reduced anxiety. Nat Genet *23*, 99-103.
Tronche, F., Kellendonk, C., Reichardt, H. M., and Schutz, G. (1998). Genetic dissection of glucocorticoid receptor function in mice. Curr Opin Genet Dev *8*, 532-538.
Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A. S., Sieber, B. A., Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., and Arumae, U. (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature *381*, 785-789.
Tsankova, N. M., Kumar, A., and Nestler, E. J. (2004). Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. J Neurosci *24*, 5603-5610.
Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., and Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. Cell *87*, 1317-1326.
Tsou, A. P., Kosaka, A., Bach, C., Zuppan, P., Yee, C., Tom, L., Alvarez, R., Ramsey, S., Bonhaus, D. W., Stefanich, E., and et al. (1994). Cloning and expression of a 5-hydroxytryptamine7 receptor positively coupled to adenylyl cyclase. J Neurochem *63*, 456-464.
Tsukada, T., Fink, J. S., Mandel, G., and Goodman, R. H. (1987). Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J Biol Chem *262*, 8743-8747.
Turnbull, A. V., and Rivier, C. (1997). Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc Soc Exp Biol Med *215*, 1-10.
Turnbull, A. V., and Rivier, C. L. (1999). Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol Rev *79*, 1-71.
Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science *213*, 1394-1397.
Van den Veyver, I. B. (2002). Genetic effects of methylation diets. Annu Rev Nutr *22*, 255-282.
Van Eekelen, J. A., Jiang, W., De Kloet, E. R., and Bohn, M. C. (1988). Distribution of the mineralocorticoid and the glucocorticoid receptor mRNAs in the rat hippocampus. J Neurosci Res *21*, 88-94.
van Leengoed, E., Kerker, E., and Swanson, H. H. (1987). Inhibition of post-partum maternal behaviour in the rat by injecting an oxytocin antagonist into the cerebral ventricles. J Endocrinol *112*, 275-282.
Vayssiere, B. M., Dupont, S., Choquart, A., Petit, F., Garcia, T., Marchandeau, C., Gronemeyer, H., and Resche-Rigon, M. (1997). Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. Mol Endocrinol *11*, 1245-1255.
Villescas, R., Bell, R. W., Wright, L., and Kufner, M. (1977). Effect of handling on maternal behavior following return of pups to the nest. Dev Psychobiol *10*, 323-329.
Virgin, C. E., Jr., Ha, T. P., Packan, D. R., Tombaugh, G. C., Yang, S. H., Horner, H. C., and Sapolsky, R. M. (1991). Glucocorticoids inhibit glucose transport and glutamate uptake in hippocampal astrocytes: implications for glucocorticoid neurotoxicity. J Neurochem *57*, 1422-1428.
Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetyla-

tion. Nat Genet *23*, 62-66.
Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995). A structural role for hormone in the thyroid hormone receptor. Nature *378*, 690-697.
Wakshlak, A., and Weinstock, M. (1990). Neonatal handling reverses behavioral abnormalities induced in rats by prenatal stress. Physiol Behav *48*, 289-292.
Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science *284*, 339-343.
Wang, J. H., and Kelly, P. T. (1996). The balance between postsynaptic Ca(2+)-dependent protein kinase and phosphatase activities controlling synaptic strength. Learn Mem *3*, 170-181.
Wang, S., Bartolome, J. V., and Schanberg, S. M. (1996). Neonatal deprivation of maternal touch may suppress ornithine decarboxylase via downregulation of the proto-oncogenes c-myc and max. J Neurosci *16*, 836-842.
Watanabe, T. K., Bihoreau, M. T., McCarthy, L. C., Kiguwa, S. L., Hishigaki, H., Tsuji, A., Browne, J., Yamasaki, Y., Mizoguchi-Miyakita, A., Oga, K., *et al.* (1999). A radiation hybrid map of the rat genome containing 5,255 markers. Nat Genet *22*, 27-36.
Waterland, R. A., and Jirtle, R. L. (2003). Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol *23*, 5293-5300.
Watt, F., and Molloy, P. L. (1988). Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev *2*, 1136-1143.
Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., and Meaney, M. J. (2004). Epigenetic programming by maternal behavior. Nat Neurosci *7*, 847-854.
Weaver, I. C., Champagne, F. A., Brown, S. E., Dymov, S., Sharma, S., Meaney, M. J., and Szyf, M. (2005). Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci *25*, 11045-11054.
Weaver, I. C., Grant, R. J., and Meaney, M. J. (2002). Maternal behavior regulates long-term hippocampal expression of BAX and apoptosis in the offspring. J Neurochem *82*, 998-1002.
Weaver, I. C., La Plante, P., Weaver, S., Parent, A., Sharma, S., Diorio, J., Chapman, K. E., Seckl, J. R., Szyf, M., and Meaney, M. J. (2001). Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. Mol Cell Endocrinol *185*, 205-218.
Weaver, I. C., Meaney, M. J., and Szyf, M. (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proc Natl Acad Sci U S A *103*, 3480-3485.
Weaver, I. C. G., Cervoni, N., Diorio, J., Szyf, M. & Meaney, M.J. (2001). Maternal Behavior in Infancy Regulates Methylation of the Hippocampal Glucocorticoid Receptor Promoter, In Society for Neuroscience's 31st Annual Meeting Abstracts, V. M.-Y. Lee, ed. (San Diego, C.A.: Society for Neuroscience), pp. Program # 697.615.
Weinstock, M., Fride, E., and Hertzberg, R. (1988). Prenatal stress effects on functional development of the offspring. Prog Brain Res *73*, 319-331.
Welberg, L. A., and Seckl, J. R. (2001). Prenatal stress, glucocorticoids and the program-

ming of the brain. J Neuroendocrinol *13*, 113-128.
Welberg, L. A., Seckl, J. R., and Holmes, M. C. (2000). Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. Eur J Neurosci *12*, 1047-1054.
Welberg, L. A., Seckl, J. R., and Holmes, M. C. (2001). Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behaviour. Neuroscience *104*, 71-79.
Whishaw, I. Q. (1998). Place learning in hippocampal rats and the path integration hypothesis. Neurosci Biobehav Rev *22*, 209-220.
Widnell, K. L., Chen, J. S., Iredale, P. A., Walker, W. H., Duman, R. S., Habener, J. F., and Nestler, E. J. (1996). Transcriptional regulation of CREB (cyclic AMP response element-binding protein) expression in CATH.a cells. J Neurochem *66*, 1770-1773.
Windle, R. J., Wood, S., Shanks, N., Perks, P., Conde, G. L., da Costa, A. P., Ingram, C. D., and Lightman, S. L. (1997). Endocrine and behavioural responses to noise stress: comparison of virgin and lactating female rats during non-disrupted maternal activity. J Neuroendocrinol *9*, 407-414.
Wolff, G. L., Kodell, R. L., Moore, S. R., and Cooney, C. A. (1998). Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. Faseb J *12*, 949-957.
Wong, D. L., Siddall, B. J., Ebert, S. N., Bell, R. A., and Her, S. (1998). Phenylethanolamine N-methyltransferase gene expression: synergistic activation by Egr-1, AP-2 and the glucocorticoid receptor. Brain Res Mol Brain Res *61*, 154-161.
Wood, E. R., Dudchenko, P. A., and Eichenbaum, H. (1999). The global record of memory in hippocampal neuronal activity [see comments]. Nature *397*, 613-616.
Worley, P. F., Bhat, R. V., Baraban, J. M., Erickson, C. A., McNaughton, B. L., and Barnes, C. A. (1993). Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. J Neurosci *13*, 4776-4786.
Wright, E. M., Vogel, K. S., and Davies, A. M. (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron *9*, 139-150.
Wylie, L. M., Robertson, G. W., and Hocking, P. M. (2003). Effects of dietary protein concentration and specific amino acids on body weight, body composition and feather growth in young turkeys. Br Poult Sci *44*, 75-87.
Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. Int Rev Cytol *68*, 251-306.
Xu, D., and Juliano, R. L. (2005). Epigenetic modulation of gene expression in mammalian cells. Crit Rev Eukaryot Gene Expr *15*, 93-101.
Yamamoto, I., Potts, J. T., Jr., and Segre, G. V. (1988). Glucocorticoids increase parathyroid hormone receptors in rat osteoblastic osteosarcoma cells (ROS 17/2). J Bone Miner Res *3*, 707-712.
Yan, Q., Radeke, M. J., Matheson, C. R., Talvenheimo, J., Welcher, A. A., and Feinstein, S. C. (1997). Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J Comp Neurol *378*, 135-157.
Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285-291.
Yang, S. N., Wu, J. N., Liu, D., and Tung, C. S. (1998). Metabotropic glutamate receptors

are involved in calcium-induced LTP of AMPA and NMDA receptor-mediated responses in the rat hippocampus. Brain Res Bull *46*, 505-512.
Yau, J. L., Olsson, T., Morris, R. G., Noble, J., and Seckl, J. R. (1996). Decreased NGFI-A gene expression in the hippocampus of cognitively impaired aged rats. Brain Res Mol Brain Res *42*, 354-357.
Young, E. A., Lewis, J., and Akil, H. (1986). The preferential release of beta-endorphin from the anterior pituitary lobe by corticotropin releasing factor (CRF). Peptides *7*, 603-607.
Young, L. J., Wang, Z., Donaldson, R., and Rissman, E. F. (1998). Estrogen receptor alpha is essential for induction of oxytocin receptor by estrogen. Neuroreport *9*, 933-936.
Yudt, M. R., and Cidlowski, J. A. (2001). Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol *15*, 1093-1103.
Zha, H., Fisk, H. A., Yaffe, M. P., Mahajan, N., Herman, B., and Reed, J. C. (1996). Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell Biol *16*, 6494-6508.
Zhang, Y., Ng, H. H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev *13*, 1924-1935.
Zhou, X. F., and Rush, R. A. (1996). Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. Neuroscience *74*, 945-953.
Zhu, Y., Prehn, J. H., Culmsee, C., and Krieglstein, J. (1999). The beta2-adrenoceptor agonist clenbuterol modulates Bcl-2, Bcl-xl and Bax protein expression following transient forebrain ischemia. Neuroscience *90*, 1255-1263.
Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Thoenen, H., and Lindholm, D. (1995). Characterization of TrkB receptor-mediated signaling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. J Neurochem *65*, 2241-2250.
Zong, J., Ashraf, J., and Thompson, E. B. (1990). The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. Mol Cell Biol *10*, 5580-5585.
Zwaal, R. F., and Schroit, A. J. (1997). Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood *89*, 1121-1132.